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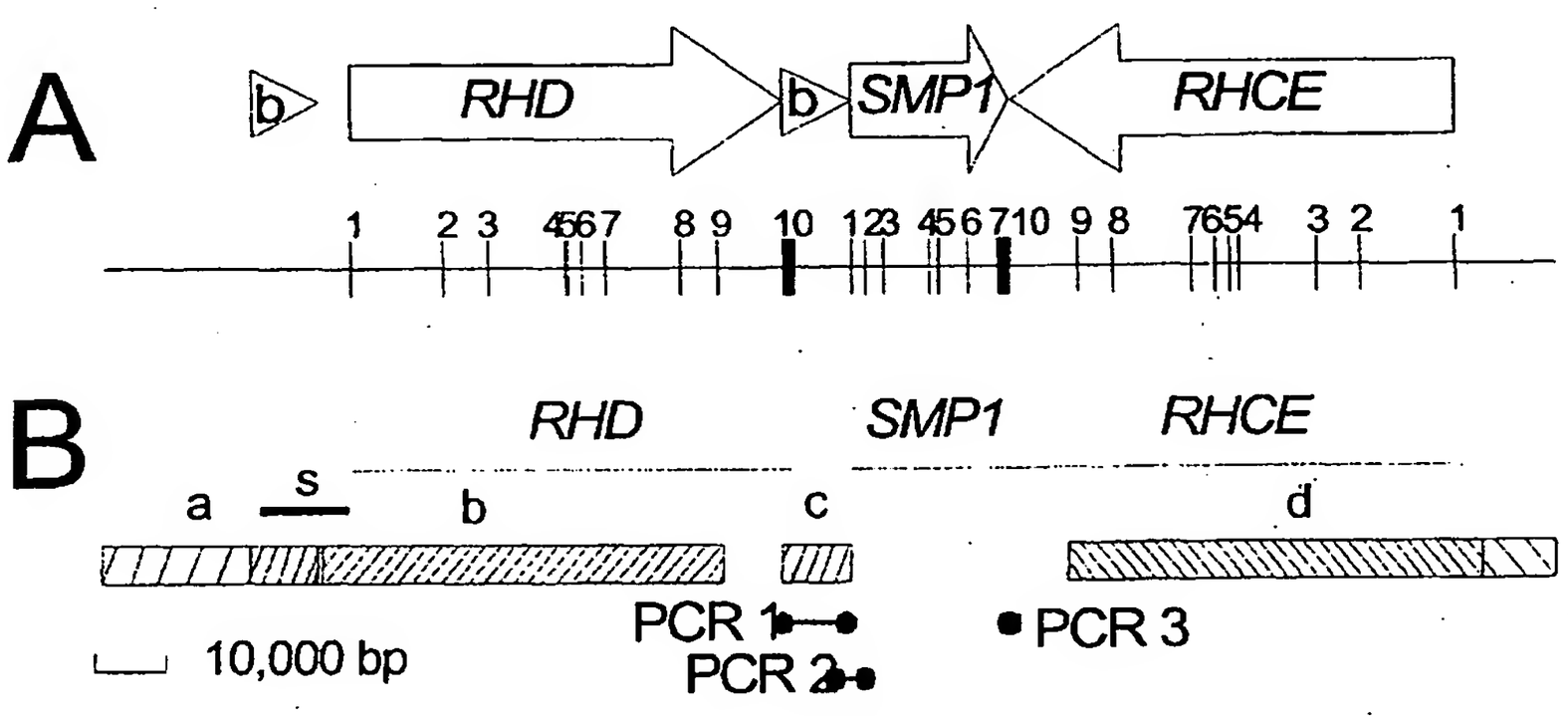
(54) Title: MOLECULAR STRUCTURE OF *(RHI)* NEGATIVE



(57) Abstract: The present invention relates to a nucleic acid molecular structure representing the Rhesus genes locus comprising the *RHD, SMP1* and *RHCE* genes and/or the *Rhesus box(es)*, preferably the hybrid *Rhesus box*, the upstream *Rhesus box* and/or the downstream *Rhesus box*. Furthermore, the invention relates to a process for the specific detection of the common *RHD* negative haplotypes. The invention further relates to the detection of *RHD* positive hyplotypes in D-negative individuals. Various mutations in the *RHD* gene have been identified that allow for the development of diagnostic tools. The invention also relates to oligonucleotides, that specifically hybridize to the hybrid box, preferably the breakpoint or breakpoint region or to the upstream and downstream *Rhesus boxes*. Additionally, the invention relates to kits comprising or employing the above recited compounds of the invention.

WO 01/32702 A2

MOLECULAR STRUCTURE OF *(RHI)* NEGATIVE

The present invention relates to a nucleic acid molecular structure representing the Rhesus genes locus comprising the *RHD, SMP1* and *RHCE* genes and/or the *Rhesus box*(es), preferably the hybrid *Rhesus box*, the upstream *Rhesus box* and/or the downstream *Rhesus box*. Furthermore, the invention relates to a process for the specific detection of the common *RHD* negative haplotypes. The invention further relates to the detection of *RHD* positive haplotypes in D-negative individuals. Various mutations in the *RHD* gene have been identified that allow for the development of diagnostic tools. The invention also relates to oligonucleotides, that specifically hybridize to the hybrid box, preferably the breakpoint or breakpoint region or to the upstream and downstream *Rhesus boxes*. Additionally, the invention relates to kits comprising or employing the above recited compounds of the invention.

Several documents are cited throughout the text of this specification; the disclosure content of each of the documents cited herein (including any manufacturer's specifications, instructions, etc.) is hereby incorporated by reference.

The Rhesus D antigen (ISBT 004.001; RH1) is the most important blood group antigen determined by a protein. Anti-D remains the leading cause of hemolytic disease of the newborn (Filbey, Acta Obstet Gynecol Scand, 74:687,1995; Bowman, J, Semin Perinatol 21:39, 1997). Depending on the population, 3% to 25% of whites lack the antigen D (Mourant, The distribution of the human blood groups and other polymorphisms, London, Oxford University Press, 1976). Anti-D immunizations can occur readily in D-negative recipients (Urbaniak, Transfusion 21:64, 1981).

The antigens of the RH blood group are carried by proteins coded by two genes, *RHD* and *RHCE*, that are located at chromosomal position 1p34.1 - 1p36 (Cherif-

Zahar, Hum. Genet. 86: 398, 1991; MacGeoch, Cytogenet. Cell Genet. 59:261, 1992) probably within less than a 450,000 base pair (bp) distance (Carritt, Hum. Mol. Genet. 6:843, 1997). Both genes encompass ten exons and their structures are highly homologous. The relative orientation of the genes, their distance, and the possibility of interspersed other genes were unknown (Flegel, Transfus. Med. 8:281, 1998). Very recently, Okuda et al. (Okuda, Biochem. Biophys. Res. Commun. 263:378, 1999) reported a sequence of about 11,000 bp, which was thought to represent the DNA segment between *RHD* and *RHCE*.

In whites, the vast majority of D-negative haplotypes is due to a deletion of the *RHD* gene: This deletion spans the whole *RHD* gene, because *RHD*-specific sequences ranging from exon 1 to the 3' untranslated region are absent (Gassner, Transfusion 37:1020, 1997). The exact extent of the deletion was uncertain, leaving open the possibility that neighboring genes were also affected.

The identification of the *RHD* gene as the molecular basis of the D antigen allowed RhD phenotype prediction by DNA typing (Flegel, Transfus. Med. 8:281, 1998; Lo, Lancet 341:1147, 1993). However, since the structure of the prevalent D-negative haplotype is unknown, a specific detection of the *RHD* deletion remained impossible and the discrimination of RHD^+/RHD^+ homozygous from RHD^+/RHD^- heterozygous individuals relied on indirect methods. This discrimination is of clinical interest in particular, because in D-negative mothers with an anti-D, the risk of an affected child is 100% with a RHD^+/RHD^+ father, but only 50% with a RHD^+/RHD^- father.

Several indirect approaches have been applied to determine the zygosity: (i) a simple guess based on the phenotype is correct in about 95% of cases, (ii) determination of the D antigen density which can be confounded by factors such as the presence of the C antigen, and (iii) several methods involving the parallel quantitative amplification of *RHD*- and *RHCE*-specific sequences (Cossu, Electrophoresis 17:1911, 1996; Döscher, Infusionsther. Transfusionsmed. 26(suppl 1):31, 1999 (abstr.)). These elaborate techniques may not be practical in routine laboratories. In addition, several investigators identified polymorphisms in the *RHCE* gene or neighboring sequences genetically linked to the lack of the *RHD* gene (Carritt, Hum. Mol. Genet. 6:843, 1997; Huang, Am. J. Hum. genet. 58: 133,

1996; Fujiwara, Hum. genet. 104:301, 1999; Onda, Gene 159:225,1995). This indirect approach relied on the linkage disequilibrium associating the *RHD* deletion with a polymorphism.

Furthermore, the utility of the *RHD* PCR is limited by the incomplete knowledge of presumably rare *RHD* positive alleles in RhD-negative. *RHD* positive alleles in RhD negative are caused by *RHD-CE-D* hybrid genes (Huang, Blood 88:2326-33, 1996; Faas, Transfusion 37:38-44, 1997, Faas, Transfusion 36:506-11, 1996), nonsense-mutations (Avent, Blood 89:2568-77, 1997), frameshifts (Andrews, Blood 92:1839-40, 1998; Cherif-Zahar Br. J. Haematol. 102:1263-70, 1998), or pseudogenes (Singleton, Blood 95:12-8, 2000). Such alleles are frequent in Africans (Faas, Transfusion 37:38-44, 1997, Singleton, Blood 95:12-18, 2000) and Asians (Okuda, J. Clin. Invest. 100:373-9,1997) but rare in whites. Nevertheless, recent analyses (Avent, Blood 89:2568-77, 1997; Flegel, Transfus. Med. 8:281-302, 1998) suggested that even for whites these alleles are likely the leading cause of incorrect Rh phenotype prediction. Several observations in whites (Avent, Blood 89:2568-77, 1997; Hyland, Blood 84:321-4, 1994) indicated that these alleles clustered in the Cde and cdE haplotypes.

The most direct approach for analyzing the *RHD* locus on the molecular level would be PCR amplification spanning the *RHD* deletion site. Such an assay has, so far, not been available because the structure of the *RHD* locus in RhD positives and RhD negatives was incompletely understood.

Accordingly, the technical problem underlying the present invention was to provide means and methods for a reliable, nucleic acid based analysis of the Rhesus D locus. These means and methods should be, inter alia, suitable for the detection and/or discrimination of RHD^+/RHD^+ and RHD^+/RHD^- individuals.

The solution to said technical problem is achieved by providing the embodiments characterized in the claims.

Thus, the invention relates to a nucleic acid molecular structure representing the *Rhesus* genes locus comprising the *RHD, SMP1,* and *RHCE* genes and/or the

Rhesus boxes, preferably the hybrid *Rhesus box*, the upstream *Rhesus box* and/or the downstream *Rhesus box*, the sequences of which are shown in Figures 8 to 10.

In the context of the present invention, the term "nucleic acid molecular structure" is defined as a linear DNA-segment that comprises, in its broadest meaning, the combination of the above mentioned genes, namely the *RHD, SMP1* and *RHCE* genes, arranged in this 5‘ to 3‘ order and/or *Rhesus boxes* that co-determine said *Rhesus* gene locus. DNA sequences that give rise to the molecular structure of the invention include the following: The nucleotide sequence structure consists of a *Rhesus box* 5' flanking region, the hybrid *Rhesus box* or two *Rhesus boxes* with intervening *RHD* gene, and the *Rhesus box* 3' flanking region.

The following sequences represent preferred embodiments contained in the nucleic acid molecular structure of the invention.

The *Rhesus box* 5' flanking region is represented in the genomic clone HS465N24 (GenBank accession number AL031432.1), bases 1 to 120,156.

The hybrid *Rhesus box* is represented in GenBank accession number AL252313 bases 33 to 9,180.

The two *Rhesus boxes* with intervening *RHD* gene consists of the upstream *Rhesus box*, represented in GenBank accession number AL252311 bases 34 to 9,175, the *RHD* gene and the downstream *Rhesus box* represented in GenBank accession number AL252312 bases 23 to 9,177 (see Fig. 8 to 10).

The *Rhesus box* 3' flanking region consists of a small DNA segment between the downstream or hybrid *Rhesus box* and the *SMP1* gene, the *SMP1* gene and the *RHCE* gene.

The *RHD* gene consists of a *RHD* 5' region homologous to genomic clone HS469D22 (GenBank accession number AL031284.9) bases 56,012 to 51,472;

also represented by a nucleotide segment dubbed "stuffer fragment" (GenBank accession number AB029152) bases 7,716 to 11,005; the *RHD* promoter (GenBank accession number AJ252314) bases 1 to 1,246 (see Figure 11) and the *RHD* gene defined by the *RHD* cDNA (GenBank accession number X63097) bases 1 to 1,371 and by its intron sequences.

The small DNA segment preferably comprises 15 nucleotides between the downstream or hybrid *Rhesus box* and the *SMP1* gene and is represented in AL252312 by bases 9,178 to 9,192.

The *SMP1* gene is defined by the *SMP1* cDNA represented in GenBank accession number AF0811282 and by its intron sequences.

The *RHCE* gene is defined by the *RHCE* cDNA represented in GenBank accession number X63095 and by its intron sequences and further represented in part by the genomic clone HS469D22 (GenBank accession number AL031432.1) bases 1 to 51,471 and the *RHCE* 5' flanking region represented by genomic clone HS469D22 bases 51,472 to 84,811.

Whereas the upstream *Rhesus box* is located 5' of the *RHD* gene, the downstream *Rhesus box* is located between the *RHD* and *SMP1* genes in this structure of the present invention. Alternatively, the term "nucleic acid molecular structure" relates to DNA segments solely comprising the referenced *Rhesus boxes*. This term, in a further alternative, relates to DNA segments comprising the *RHD, SMP1* and *RHCE* genes and two *Rhesus boxes*, namely the upstream *Rhesus box* and the downstream *RHD* box. Comprised by this term are also, in a further alternative, DNA segments that comprise the hybrid *Rhesus box*, the *SMP1* gene and the *RHCE* gene. In another alternative, the term relates to DNA segments comprising the *SMP1* gene and the hybrid *Rhesus box*. This term in a further alternative relates to DNA segments comprising the upstream *Rhesus box*, *RHD*, downstream *Rhesus box* and *SMP1*.

This term in another alternative relates to DNA segments comprising the downstream *Rhesus box* and *SMP1*. For a better understanding of the claimed subject-matter, it is referred to figures 1 and 7, infra.

In accordance with the present invention, the term "nucleic acid molecular structure" comprises also any feasible derivative of the above referenced nucleic acid structure to which a nucleic acid probe may hybridize. In other words, the structure of the invention may be prepared by synthetic or semisynthetic means and thus consist of or comprise peptide nucleic acid. Said term also bears the meaning of a nucleic acid molecule.

In accordance with the present invention, the term "*Rhesus box*" describes upstream and downstream DNA segments that flank the *RHD* gene on the 5' and 3' end. The three *Rhesus boxes* are defined by their nucleotide sequences. The hybrid *Rhesus box* is represented in one embodiment in GenBank accession number AL252313 bases 33 to 9,180. The two *Rhesus boxes* with intervening *RHD* gene consists of the upstream *Rhesus box*, represented in one embodiment in GenBank accession number AL252311 bases 34 to 9,175 and the downstream *Rhesus box* represented in one embodiment in GenBank accession number AL252312 bases 23 to 9,177. As exemplified in the appended examples the *Rhesus boxes* are preferably approximately 9000bp long, having 98,6% identity and identical orientation. According to the present invention the upstream and downstream *Rhesus boxes* are at least 95% homologous. The length of these *Rhesus boxes* may vary. It is expected that the length of these *Rhesus boxes* may vary, because, among other structural features, multiple repetitive elements, some of them are organized in tandem arrays, are known to be prone to (array) elongation and deletion events. If such events occur the length of the *Rhesus boxes* may shrink to less than 1,000 nucleotides length or extend to more than 20,000 nucleotides length.

In accordance with the present invention the term "identity" refers to the determination of sequence identity using suitable alignment programs, such as BLAST.

As has been pointed out above, the diagnostic analysis of *RHD* negatives on the molecular level has so far been hampered by the fact, that the overall structure of the *RHD/RHCE* loci was unknown. It has now been surprisingly found, that the two genes, *RHD* and *RHCE*, have opposite orientation and face each other with their 3' ends. In accordance with the present invention it has further been found that the *RHD* gene is surrounded by two highly homologous *Rhesus boxes*. The physical distance between *RHD* and *RHCE* is about 30,000 bp and is filled with a *Rhesus box* and the *SMP1* gene. The breakpoints of the *RHD* deletion in the prevalent *RHD* negative haplotypes are located in the 1,463 bp identity region of the *Rhesus boxes*. Similar *RHD* deletion events may involve any other region within the highly homologous *Rhesus boxes*. Hence, a region of a breakpoint comprising an *RHD* deletion other than the common *RHD* deletion may be anticipated to occur anywhere within the *Rhesus boxes* as defined above.

The opposite orientation of the two *RH* genes explains the different character of hybrid genes in the MNS and RH blood group: The glycophorin genes encoding the MNS antigens occur in the same orientation (Onda, Gene 159:225, 1995), and many recombinations may be explained as unequal crossing over resulting in single hybrid genes (Blumenfeld, Hum. Mutat. 6:1999, 1995). Based on the surprising findings referred to above, the events on the molecular level that lead to *RHD* negatives can now be more fully understood. In the *RH* locus, the inversely oriented sequences are unlikely to trigger unequal crossing over, and if this event occurred, no functional hybrid gene would result. The conclusion that unequal crossing over at the *RH* gene locus is unlikely may explain that most *RH* hybrid genes are of *RHD-CE-D* or *RHCE-D-CE* type and involve stretches of homologous DNA positioned *in cis* as noted previously (Wagner, Blood 91:2157, 1998). Currently, the *RH* gene system is the only well investigated gene locus where the two genes have opposite orientation, rendering it a model system for the evolution of neighboring, oppositely oriented genes that are frequent throughout genomes.

Based on the structure of the *RH* gene locus (Fig. 1), a parsimonious model for the *RHD* gene deletion event is proposed (Fig. 7). Although the applicant does not

wish to be bound to theory, the following is believed with regard to the generation of RhD negative. The *RHD* deletion may be explained by unequal crossing over triggered by the highly homologous *Rhesus boxes* embracing the *RHD* gene. The hybrid-type *Rhesus box* of *RHD*-negatives arises, when a crossover leading to a deletion event involving a breakpoint region within the identity region of the upstream and downstream *Rhesus boxes* takes place. Thus, the hybrid *RHD* box is characterized by a 5' portion derived from the upstream *RHD* box fused to a 3' portion from the downstream *RHD* box. In one preferred embodiment the breakpoint region is 903bp long. The sequence of this preferred hybrid *Rhesus box* is depicted in figure 5. In the specific embodiments described in the examples, said 903 bp breakpoint region in the *Rhesus boxes* is located in a 1,463 bp stretch of 99.9% homology resembling a THE-1B human transposable element and a L2 repetitive DNA element (Fig. 4). Interestingly, the >60,000 bp DNA segment that is deleted in the *RHD* negative haplotype consisted only of and contained all sequences that are duplicated in the *RHD* positive haplotype.

The findings of the present invention referred to herein above allow for the establishment of a number of easy to do or refined methods for the analysis of the genotype of an individual with regard to the *RH* gene locus. Examples of such methods are provided herein below.

While the molecular mechanism resulting in the prevalent *RHD* negative haplotype is now apparent, it is less clear how the much older duplication event gave rise to the structure of the *RH* genes in *RHD* positives. The duplication of the *Rhesus box* and the *RH* genes probably occurred as a single event, because the overall homology of the two *Rhesus boxes* is very similar to that of the *RH* genes. Without being bound by theory, it is tempting to speculate that the *RHD* duplication originate in causal connection with the insertion of the near full-length THE-1B transposon-like human element in duplicate. However, the open reading frame of the THE-1B element probably was non-functional at the time of the duplication.

In a preferred embodiment of the present invention, said nucleic acid molecular structure is representative of the common *RHD* negative haplotypes.

According to the present invention, the term „is representative of" relates to a nucleic acid molecular structure comprising all sequential and structural features to relate said structure to a group of molecular structures sharing said features. In the above preferred embodiment, said features give rise to the common *RHD* negative haplotype. In the present context this means preferably the deletion of the *RHD* gene encompassing the whole *RHD* gene and its 5' region, which are located between the upstream *Rhesus box* and the downstream *Rhesus box*.

In the present context this could also mean, for example, that all structures sharing a nonsense mutation, missense mutation, splice site mutation, partial deletion, partial insertion, partial inversion or a combination thereof within the *RHD* gene, which terminates or obliterates the expression of a protein product of the *RHD* gene, are representative of the *RHD* negative haplotype.

The term "haplotype" relates to a series of linked alleles within a defined region on a single maternal or paternal chromosome.

The term "common *RHD* negative haplotype" refers to any RhD antigen negative haplotype that comprises a hybrid *Rhesus box*. Preferably the DNA segment encompassing the whole *RHD* gene and its 5' region, which are located between the upstream *Rhesus box* and the downstream *Rhesus box*, is deleted.

In another embodiment, the invention relates to a nucleic acid molecular structure, dubbed *Rhesus box*, which is flanking the breakpoint region of the *RHD* deletion in the common *RHD* negative haplotypes.

In accordance with the present invention the term "breakpoint region of the *RHD* deletion" describes a distinct DNA segment that is involved in an *RHD* deletion. As has been pointed out above, said deletion may be the result of an unequal crossing over event involving both the upstream and downstream *Rhesus boxes*, deleting interspersed sequences and finally giving rise to a nucleic acid molecular structure (the referenced *Rhesus boxes* for a better delimitation from the upstream and

downstream *Rhesus box* also referred to as hybrid *Rhesus box*) wherein the 5' portion of the upstream *RHD* box is in close spatial proximity to the 3' portion of the downstream *RHD* box. As mentioned above and depicted in figures 7 and 4 this region can preferably be 903bp long and be located in a 1,463bp stretch within the *Rhesus boxes*, having 99,9 % homology in this segment. In another preferred alternative said region is located downstream from said 903 bp fragment but is still contained within the 1463 bp stretch. Preferably, said fragment is 556 to 560bp long. The actual breakpoint may vary such that the contribution of the upstream *Rhesus box* and the downstream *Rhesus box* are different in different individuals. However, in accordance with the present invention, the breakpoint in any case occurs in the upstream and downstream *Rhesus boxes*.

The hybrid *Rhesus box* is particularly useful for the analysis of *RHD*-negative haplotypes. For example, oligonucleotides may be employed that hybridize to nucleic acid sequences comprising the breakpoint which arose as a result of the *RHD* deletion. It is to be understood that such oligonucleotides need to hybridize to a significant portion preferably encompassing 20 nucleotides, that is located 5' and 3' of the region of the actual breakpoint in order to be indicative of a deletion event. For example, when such an oligonucleotide is hybridized under stringent conditions such as 0.2 x SSC, 0.1 SDS at 65°C and the probe would be 943 nucleotides long, then the hybridizing region should include portions that hybridize 3' as well as 5' of the breakpoint.

For example, a *Rhesus box* or a part thereof encompassing the region of the breakpoint is amplified. Thereafter the amplification product is assayed in a sequence specific way by hybridization to an oligonucleotide of about six or more nucleotides length.

Preferably, a stretch of DNA representative of a *Rhesus box* or part thereof encompassing the region of the breakpoint is amplified using two primers. One primer may be located in the *Rhesus box* 5` of the identity region and is specific for both the upstream *Rhesus box* and the hybrid *Rhesus box*. The other primer may be located in the *Rhesus box* 3`of the identity region and is specific for both the

downstream *Rhesus box* and the hybrid *Rhesus box*. in this application, the presence of an amplification product of the expected size is indicative of the presence of a hybrid *Rhesus box* and hence, of the *RHD* deletion.

Another possible combination of primers is the following: One primer may be located in the *Rhesus box* 5'of the identity region and is specific for both the upstream *Rhesus box* and the hybrid *Rhesus box*. The other primer may be located in the *Rhesus box* 3'of the identity region. In this application, the presence of a hybrid *Rhesus box* is determined by examining the specificity of the parts of the amplification product pertaining to a DNA stretch of the *Rhesus box* 3'of the identity region. This may for example be effected by hybridization with an oligonucleotide that hybridizes to the hybrid *Rhesus box* and to the downstream *Rhesus box* but not to the upstream *Rhesus box*, or by digestion with a restriction enzyme that cuts the hybrid *Rhesus box* and the downstream *Rhesus box* but does not cut the upstream *Rhesus box*, or by digestion with a restriction enzyme that does not cut the hybrid *Rhesus box* and the downstream *Rhesus box* but cuts the upstream *Rhesus box*, or by nucleotide sequencing.

Another possible combination of primers is the following: One primer may be located in the *Rhesus box* 5'of the identity region. The other primer may be located in the *Rhesus box* 3'of the identity region and is specific for both the downstream *Rhesus box* and the hybrid *Rhesus box*. In this application, the presence of a hybrid *Rhesus box* is determined by examining the specificity of the parts of the amplification product pertaining to a DNA stretch of the *Rhesus box* 5'of the identity region. This may for example be effected by hybridization with a nucleotide that hybridizes to the hybrid *Rhesus box* and to the upstream *Rhesus box* but not to the downstream *Rhesus box*, or by digestion with a restriction enzyme that cuts the hybrid *Rhesus box* and the upstream *Rhesus box* but does not cut the downstream *Rhesus box*, or by digestion with a restriction enzyme that does not cut the hybrid *Rhesus box* and the upstream *Rhesus box* but cuts the downstream *Rhesus box*, or by nucleotide sequencing.

The hybrid *Rhesus box* may also serve as a diagnostic tool for the presence of the *RHD* deletion when analyzed by an anti-DNA antibody specific for one or more embodiments of the hybrid box, a fragment or derivative thereof such as an scFvFab or $F(ab')_2$ fragment or an aptamer etc. Thus, antibodies, fragments or derivatives thereof or such aptamers can be generated by the person skilled in the art according to conventional technology (see, for example, Harlow and Lane, "Antibodies, A Laboratory Manual", CSH Press 1988, Cold Spring Harbor).

In a preferred embodiment, the invention relates to a nucleic acid molecular structure representative of an *RHD* negative haplotype comprising an *RHD* gene deletion involving the upstream *Rhesus box*, the downstream *Rhesus box*, or both.

The invention further relates to a nucleic acid molecular structure that is flanking the *Rhesus box* in the common *RHD* negative haplotypes.These structures or sequences can be used to derive primers for amplification reactions such as long range PCR for the molecular analysis of the *RHD* locus.

For example, a stretch of DNA representative of a *Rhesus box* and parts of their flanking regions or parts thereof encompassing the region of the breakpoint is amplified using two primers. One primer may be located in the 5'flanking region of the *Rhesus box*. Alternatively, this primer may be located in the *Rhesus box* 5'of the identity region and is specific for both the upstream *Rhesus box* and the hybrid *Rhesus box*. The other primer may be located in the *Rhesus box* 3'flanking region. Alternatively, this primer may be located in the *Rhesus box* 3'of the identity region and is specific for both the downstream *Rhesus box* and the hybrid *Rhesus box*. In this application the presence of an amplification product of the expected size is indicative of the presence of a hybrid *Rhesus box* and hence, of the *RHD* deletion.

Another possible combination of primers is the following: One primer may be located in the 5'flanking region of the *Rhesus box*. The other primer may be located in the *Rhesus box* 3' of the identity region. In this application, the presence of a hybrid *Rhesus box* is determined by examining the specificity of the parts of the amplification product pertaining to a DNA stretch of the *Rhesus box* 3'of the

identity region. This may for example be effected by hybridization with an oligonucleotide that hybridizes to the hybrid *Rhesus box* and to the downstream *Rhesus box* but not to the upstream *Rhesus box*, or by digestion with an restriction enzyme that cuts the hybrid *Rhesus box* and the downstream *Rhesus box* but does not cut the upstream *Rhesus box*, or by digestion with a restriction enzyme that does not cut the hybrid *Rhesus box* and the downstream *Rhesus box* but cuts the upstream *Rhesus box*, or by nucleotide sequencing.

Another possible combination of primers is the following: One primer may be located in the *Rhesus box* 5' of the identity region. The other primer may be located in the 3' flanking region of the *Rhesus box*. In this application, the presence of a hybrid *Rhesus box* is determined by examining the specificity of the parts of the amplification product pertaining to a DNA stretch of the *Rhesus box* 5'of the identity region. This may for example be effected by hybridization with an oligonucleotide that hybridizes to the hybrid *Rhesus box* and to the upstream *Rhesus box* but not to the downstream *Rhesus box*, or by digestion with an restriction enzyme that cuts the hybrid *Rhesus box* and the upstream *Rhesus box* but does not cut the downstream *Rhesus box*, or by digestion with a restriction enzyme that does not cut the hybrid *Rhesus box* and the upstream *Rhesus box* but cuts the downstream *Rhesus box*, or by nucleotide sequencing.

In a preferred embodiment the nucleic acid molecular structure is representative of *RHD* positive haplotypes.

The term „*RHD* positive haplotype" refers to any haplotype that comprises DNA sequences specific for the *RHD* gene.

In a preferred embodiment the invention relates to a nucleic acid molecular structure respresentative of the common *RHD* positive haplotype.

In another preferred embodiment the nucleic acid molecular structure is derived from a sample comprising an *RHD* positive haplotype that is serologically classified RhD negative.

In the context of the invention, the term "serologically classified RhD negative" describes a sample that has been tested for the presence of RhD antigen using, e.g., routine serological assays wherein the result of such assays was negative.

In a particularly preferred embodiment the sample that is classified RhD negative is obtained from a Caucasian population.

In another more preferred embodiment the nucleic acid molecular structure comprises a partial *RHD* deletion.

One alternative for explaining that an allele routinely diagnosed *RHD* positive gives rise to a RhD-negative phenotype is the deletion of a part of the *RHD* gene or the substitution of a part of the *RHD* gene by the corresponding DNA segments derived from the *RHCE* gene not detected by standard diagnostic methods such as PCR.

In a preferred embodiment said deletions or substitutions comprise *RHD* exons 3 to 7 or 4 to 7 giving rise to a CcddEe phenotype, or 1 to 9.

In a most preferred embodiment said substitution comprises a *RHD-CE(3-7)-D* hybrid gene, a *RHD-CE(4-7)-D* hybrid gene giving rise to a CcddEe phenotpye or a *RHCE(1-9)-D(10)* hybrid gene, all of which correlate with a RhD negative phenotype.

In another most preferred embodiment the nucleic acid molecular structure of the present invention comprising an *RHD-CE-D* hybrid allele, which is representative of a Cde^s haplotype but also occurs in other Rhesus haplotypes, carrying a 5' breakpoint region located in intron 3, the sequence of which breakpoint region is shown in Figure 12, and/or a 5' breakpoint region located in intron 7, the sequence of which breakpoint region is shown in Figure 13, or both breakpoint regions.

Cde^{s}, also known as r'^{s}, is a *RH* haplotype resembling *Cde* that was initially characterized as expressing antigen e^{s} instead of antigen e, expressing antigen c, and expressing reduced and altered antigen C (Issitt, P.D. *Aplied Blood Group Serology*, Miami: Montgomery Scientific Publications, 1985, page 239). The molecular structure underlying this haplotype has recently been elucidated (Blunt, T., Daniels, G., and Carritt, B. Serotype switching in a partially deleted *RHD* gene. *Vox Sang.* 67:397-401, 1994; Faas, B.H.W., Becker, E.A.M., Wildoer, P., Ligthart, P.C., Overbeeke, M.A.M., Zondervan, H.A., von dem Borne, A.E.G.K., and van der Schoot, C.E. Molecular background of VS and weak C expression in blacks. *Transfusion* 37:38-44, 1997; Daniels, G.L., Faas, B.H., Green, C.A., Smart, E., Maaskant-van Wijk, P.A., Avent, N.D., Zondervan, H.A., von dem Borne, A.E., and van der Schoot, C.E. The VS and V blood group polymorphisms in Africans: a serologic and molecular analysis. *Transfusion* 38:951-958, 1998.): The *Cde*^{s} haplotype contains a *RHD-CE-D* hybrid gene encoding for an antigen C immunoreactivity, in which exons 4 to 7 derived from *RHCE* and exon 3 has a *RHD* like structure but possesses a *RHCE* specific Thr at codon 152.

Several additional *RHD* positive alleles occurring in RhD negative individuals have previously been partly or fully characterized (Table 10). Three of these ten published *RHD* alleles represented *RHD-CE-D* hybrid alleles in which the *RHCE* specific stretch encompassed at least exons 4 to 7. For each of these three hybrid *RHD* alleles, alleles were found whose patterns would be compatible (Table 10). Out of the seven RhD negative patterns observed in the present study, six were compatible with such type of hybrid *RHD* allele. Seven out of ten published *RHD* alleles represented deletions, nonsense mutations or a pseudogene. None of these alleles occurred in this study, which may indicate that they are rare in whites. In another embodiment, the invention provides for the detection or determination of the *RHD* allele previously described as *RHD* exon 9 negative (Gassner, Transfusion 37:1020ff, 1997), preferably representing an hybrid *RHD-CE(9)-D* hybrid allele, by its lack of *RHD* specific sequences in parts of intron 7 and intron 8. The specific steps carried out in this method may be any of the steps referred to in

the further methods of the invention described in the specification, alone or in any combination.

In a particularly preferred embodiment the nucleic acid molecular structure of the present invention wherein the *RHD-CE-D* hybrid of the present invention encodes a polypeptide having antigen C reactivity.

Antigen C is a blood group antigen belonging to the RH blood group system known in the art and designated as 004.002 according to the ISBT nomenclature. A description is contained in many textbooks on immunohematology, e.g. Reid, M.E. and Lomas-Francis, C. *The Blood Group Antigen Facts Book*, San Diego: Academic Press, 1997.

Furthermore, in a preferred embodiment of the invention the nucleic acid molecular structure of the invention or a nucleic acid molecule being derived from the *RHD* gene comprises a single nucleotide substitution within the coding region of the *RHD* gene or within a 5' or 3' splice site.

The term "a nucleic acid molecule derived from the *RHD* gene" is intended to mean that this nucleic acid molecule originates from the *RHD* gene but carries a mutation, deletion, insertion, substitution or duplication within the coding region, any of the splice sites or a non-coding region. Preferably, said nucleic acid molecule gives rise to an aberrant polypeptide.

In a further preferred embodiment said nucleotide substitution gives rise to a stop-codon at codon 16.

In a more preferred embodiment said substitution gene gives rise to an *RHD(W16X)* mutation.

In an additional more preferred embodiment said substitution is a G→A substitution at nucleotide position 48.

In a further preferred embodiment of the invention said nucleotide substitution gives rise to a stop codon at codon 330.

In a more preferred embodiment of the invention said substitution gives rise to a *RHD*(Y330X) mutation.

In an even more preferred embodiment of the invention said substitution is a C → G substitution at nucleotide position 985.

In another preferred embodiment of the invention said substitution gives rise to a missense mutation at codon 212.

In another preferred embodiment of the invention said substitution gives rise to a *RHD(G212V)* missense mutation.

In a more preferred embodiment of the invention said substituion is a G→T substitution at nucleotide position 635.

In a different preferred embodiment of the invention said substitution gives rise to a mutation within a 4 nucleotide sequence, a 6-nucleotide sequence or an 8-nucleotide sequence comprising the consensus splice site at the exon 8/intron 8 boundary.

In another more preferred embodiment of the invention said substituion give rise to a *RHD(G1153(+1)A)* mutation.

In an additional more preferred embodiment of the invention said substitution is a substitution at the 5' splice site intron 8 from AGgt to AGat.

In a further more preferred embodiment the nucleic acid molecular structure of the invention or a nucleic acid molecule being derived from the *RHD* gene correlates with a RhD-negative phenotype.

In another preferred embodiment of the invention said substitution gives rise to a mutation within a 4-nucleotide, a 6-nucleotide sequence or an 8-nucleotide sequence sequence comprising the consensus splice site of the exon 3/intron3 boundary.

In a further preferred embodiment of the invention said substitution gives rise to a *RHD(G486(+1)A)* mutation.

In an additional more preferred embodiment of the invention said substitution is a substitution at the 5' splice site intron 3 from ACgt to ACat.

In a further preferred embodiment of the invention said substitution gives rise to a mutation within a 4-nucleotide sequence, a 6-nucleotide sequence or an 8-nucleotide sequence comprising the consensus splice site of exon 9/intron 9 boundary.

In another preferred embodiment said substitution gives rise to a *RHD(K409K)* mutation.

In an additional more preferred embodiment of the invention said substitution is a substitution at the 5' splice site intron 9 from AGgt to AAgt.

In a more preferred embodiment of the invention the nucleic acid molecular structure of the invention or a nucleic acid molecule being derived from the *RHD* gene correlates with a D_{el}-phenotype.

In summary and referring to the above, *RHD* positive alleles can harbour single nucleotide substitutions leading to termination or reduction of the D-antigen expression. Using the improved detection methods disclosed in the present invention four *RHD* positive alleles in RhD negatives were found that had not been described previously. Two alleles, *RHD*(W16X) and *RHD*(Y330X) harbored stop codons preventing the expression of the full RhD protein. In three alleles, splice site mutations were found that may prevent correct splicing and RhD expression.

These alleles typed *RHD* positive in all *RHD* PCR methods tested, and a correct antigen D prediction necessitates a specific detection of these alleles or of polymorphisms linked to these alleles.

Previously, the discrimination of *RHD* homozygotes from *RHD* heterozygotes was difficult. The prevalent *RHD* negative allele could not be detected specifically (Flegel, Transfus. Med. 8:281, 1998; Cossu, Electrophoresis 17:1911, 1996). The above defined mutation found in accordance with the present invention provides the basis for the detection of the prevalent *RHD* negative haplotypes, and hence true *RHD* genotyping is now feasible.

The invention also relates to a process to specifically detect a *RHD* negative haplotype in a sample by utilizing any structural feature or nucleotide sequence or both of the above-described nucleic acid molecular structure or combinations thereof with techniques known in the art, preferably amplification reactions, such as polymerase chain reaction (PCR), more preferably by PCR-RFLP, PCR-SSP or long-range PCR.

The described PCR-RFLP and long-range PCR methods utilize either *Rhesus box* sequences or *Rhesus box* flanking sequences. By utilizing the same DNA stretches or combinations thereof, other methods, like PCR-SSO or biochips, can be developed or applied.

In one preferred embodiment of the present invention said process to specifically detect a common *RHD* negative haplotype comprises the following steps:

(a) isolating the DNA from a blood sample or blood donor;

(b) hybridizing at least two oppositely oriented primers under stringent conditions to the DNA so as to carry out a PCR;

(c) amplifying the target sequence;

(d) separating the amplification products on a gel; and

(e) analyzing the amplicons.

Said sample may or may be derived from blood, serum, sputum, feces or other body fluid. The sample to be analyzed may be treated as to extract, inter alia, nucleic acids. The isolation of DNA from preferably EDTA- or citrate agglutinated blood samples can be carryied out by a modified salting out methods, following the standard techniques as described in Gassner, Transfusion 37: 1020, 1997. The primers are preferably oligonucleotides that either occur naturally or in a purified restriction digest or are produced synthetically. The primers are preferably single stranded for a maximum of efficiency in the method of the present invention, and are preferably oligodeoxyribonucleotides. Purification of said primers is generally envisaged, prior to their use in the methods of the present invention, said purification comprising High Performance Liquid Chromatography (HPLC) or Polyacrylamide gel electrophoresis (PAGE), all technologies that are well known to the skilled artisan. Amplification methods such as PCR or LCR are well known in the art and described, for example in Flegel, Transfusion Medicine 8 (1998), 281-302; Maaskant, Transfusion 38 (1998), 1015-1021 and Legler, Transfusion (1996), 426-31.

According to the present invention a preferred method to detect the *RHD* deletion is performing PCR-RFLP using the expand high fidelity PCR-system and non-specific primers binding 5' of the end of the *Rhesus box* identity region as well as primers specific for the downstream *Rhesus box* and binding 3' of the end of the *Rhesus box* identity region. The PCR conditions involve preferably annealing at 65°C, extension for 10 min at 68°C. Thereafter, PCR amplicons are digested with PstI for 3h at 37°C and fragments resolved using 1% agarose gel. Additional preferred methods are further described in examples 10 and 11.

Another embodiment of the invention relates to a process to specifically detect a common *RHD* negative haplotype comprising the detection of the hybrid *Rhesus box*.

The detection of the hybrid *Rhesus box* provides the practitioner with an unambiguous result as regards the nature of the corresponding *RHD* allele. If the hybrid *Rhesus box* is detected, then the *RHD* gene is deleted. Detection of the

hybrid *Rhesus box* is preferentially effected by using an oligonucleotide that specifically hybridizes to a region comprising the breakpoint. The oligonucleotide used for hybridization must directly hybridize to that breakpoint and, in addition, hybridize to at least 943 nucleotides 5' and 3' of the breakpoint. Hybridization occurs preferably under stringent conditions such as 0.2 X SSC, 0.1% SDS at 65°C. The actual breakpoint within the hybrid *Rhesus box* may vary due to the exact nature of the putative crossover event. Accordingly, the hybrid *Rhesus box* may also be detected using a number of overlapping or non-overlapping oligonucleotides used for hybridization. The hybrid *Rhesus box* may also be detected using other protocols such as restriction analysis (preferably in combination with Southern blot analysis), or PCR technology, as described herein above.

Furthermore, another embodiment of the invention relates to a process to specifically detect a common *RHD* negative haplotype comprising assessing the nucleic acid molecular structure comprising the hybrid *Rhesus box* and the flanking regions thereof.

In accordance with the present invention, assessment of the molecular nucleic acid structure comprises analysis steps such as gel electrophoresis using either agarose gels or polyacrylamide gels, treatment with restriction-enzymes, blotting techniques, such as Southern or Northern blotting or related techniques, such as fluorescence-guided detection of hybridization and other techniques known in the art.

The present invention also relates to a process to specifically detect a *RHD* negative haplotype in a sample comprising the step of detecting any of the breakpoint regions mentioned in the present invention.

In a preferred embodiment the invention relates to the above-mentioned process wherein said detection or assessment comprises the determination of the length of a nucleic acid molecule comprising the hybrid *Rhesus box* or parts thereof.

Again, this preferred embodiment of the method of the invention utilizes standard separation techniques, such as gel electrophoresis or chromatography or standard techniques of nucleotide sequencing as known to a skilled artisan. Preferably the present invention utilizes a commercially available sequencing kit and an automatic sequencing machine from Applied Biosystems (ABI 373A or ABI 377), as further described in Example 5, for this purpose.

Another preferred embodiment of the invention relates to the above-mentioned process wherein said detection or assessment is effected by using PCR-RFLP, PCR-SSP or long-range PCR or a probe specifically hybridizing to the hybrid *Rhesus box*, preferably to the breakpoint or breakpoint region depicted in Figure 4 or 5 or Figure 12 or 13, or hybridizing to the upstream or downstream *Rhesus box*, preferably by Southern blot analysis, gel electrophoresis, biochip-analysis, molecular weight determination or fluorescence.

According to the present invention the term "hybridizing to" relates to stringent or non-stringent conditions. The setting of conditions is well within the skill of the artisan and to be determined according to protocols described, for example, in Sambrook, loc. cit. or Hames and Higgins, "Nucleic acid hybridization, a practical approach", IRC Press, Oxford (1985). The detection of specifically hybridizing sequences will usually require stringent hybridizing and washing conditions such as 0.2 x SSC, 0.1% SDS at 65°C. Non-stringent hybridization conditions for the detection of homologous and not exactly complementary sequences may be set at 6x SSC, 1% SDS at 50°C or 65°C. As is well known, the length of the probe and the composition of the nucleic acid to be determined constitute further parameters of the hybridization conditions.

Furthermore, the invention relates to a vector comprising the nucleic acid molecular structure or the nucleic acid molecule of the invention.

The vector may be used for propagation and/or expression or may be designed for gene transfer or targeting purposes. Methods of producing such vectors are well

known in the art. The same holds true for cloning the nucleic acids of the mutation into said vectors, as well as the propagation of vectors in suitable hosts, etc.

The vector may particularly be a plasmid, a cosmid, a virus or a bacteriophage used conventionally in genetic engineering that comprise the nucleic acid molecule of the invention. Expression vectors derived from viruses such as retroviruses, vaccinia virus, adeno-associated virus, herpes viruses, or bovine papilloma virus, may be used for delivery of the nucleic acid molecules or vector of the invention into targeted cell populations. Methods which are well known to those skilled in the art can be used to construct recombinant viral vectors; see, for example, the techniques described in Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1989). Alternatively, the polynucleotides and vectors of the invention can be reconstituted into liposomes for delivery to target cells. The vectors containing the nucleic acid molecules of the invention can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation may be used for other cellular hosts; see Sambrook, supra.

Such vectors may comprise further genes such as marker genes which allow for the selection of said vector in a suitable host cell and under suitable conditions. Preferably, the nucleic acid molecule of the invention is operatively linked to expression control sequences allowing expression in prokaryotic or eukaryotic cells. Expression of said polynucleotide comprises transcription of the polynucleotide into a translatable mRNA. Regulatory elements ensuring expression in eukaryotic cells, preferably mammalian cells, are well known to those skilled in the art. They usually comprise regulatory sequences ensuring initiation of transcription and optionally poly-A signals ensuring termination of transcription and stabilization of the transcript. Additional regulatory elements may include transcriptional as well as translational enhancers, and/or naturally-associated or heterologous promoter regions. Possible regulatory elements permitting expression

in prokaryotic host cells comprise, e.g., the PL, lac, trp or tac promoter in E. coli, and examples for regulatory elements permitting expression in eukaryotic host cells are the AOX1 or GAL1 promoter in yeast or the CMV-, SV40- , RSV-promoter (Rous sarcoma virus), CMV-enhancer, SV40-enhancer or a globin intron in mammalian and other animal cells. Beside elements which are responsible for the initiation of transcription such regulatory elements may also comprise transcription termination signals, such as the SV40-poly-A site or the tk-poly-A site, downstream of the nucleic acid molecule. Furthermore, depending on the expression system used leader sequences capable of directing the polypeptide to a cellular compartment or secreting it into the medium may be added to the coding sequence of the polynucleotide of the invention and are well known in the art. The leader sequence(s) is (are) assembled in appropriate phase with translation, initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein, or a portion thereof, into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an C- or N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product. In this context, suitable expression vectors are known in the art such as Okayama-Berg cDNA expression vector pcDV1 (Pharmacia), pCDM8, pRc/CMV, pcDNA1, pcDNA3 (In-vitrogene), or pSPORT1 (GIBCO BRL).

Preferably, the expression control sequences will be eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells, but control sequences for prokaryotic hosts may also be used.

As mentioned above, the vector of the present invention may also be a gene transfer or targeting vector. Gene therapy, which is based on introducing therapeutic genes into cells by ex-vivo or in-vivo techniques is one of the most important applications of gene transfer. Suitable vectors and methods for in-vitro or in-vivo gene therapy are described in the literature and are known to the person skilled in the art; see, e.g., Giordano, Nature Medicine 2 (1996), 534-539; Schaper, Circ. Res. 79 (1996), 911-919; Anderson, Science 256 (1992), 808-813; Isner, Lancet 348 (1996), 370-374; Muhlhauser, Circ. Res. 77 (1995), 1077-1086; Wang,

Nature Medicine 2 (1996), 714-716; WO94/29469; WO 97/00957 or Schaper, Current Opinion in Biotechnology 7 (1996), 635-640, and references cited therein. The polynucleotides and vectors of the invention may be designed for direct introduction or for introduction via liposomes, or viral vectors (e.g. adenoviral, retroviral) into the cell. Preferably, said cell is a germ line cell, embryonic cell, or egg cell or derived therefrom, most preferably said cell is a stem cell.

Additionally, the invention relates to a non-human host transformed with the vector of the invention.

Appropriate hosts comprise transgenic animals, cells such as bacteria, yeast cells, animal, preferably mammalian cells, fungal cells or insect cells. Transformation protocols including transfection, microinjection, electroporation, etc., are also well known in the art.

Further, the invention relates to a method of producing a protein product of the *RHD* gene comprising culturing the host of the invention under suitable conditions and isolating the protein product of the *RHD* gene.

It is preferred that the protein product of the *RHD* gene is exported into the culture medium where it can be collected according to conventions/methods. The term "culturing" as used in accordance with the present invention also comprises the raising of transgenic animals. Using appropriate vectors constructions and optionally appropriate feeds, the antigen may, e.g., be isolated from milk of, e.g. transgenic cows.

The invention additionally relates to a protein product of the *RHD* gene encoded by the nucleic acid molecule of the invention or produced by the method of the invention.

Preferably, the protein is in the same way post translationaly modified and has the same chemical structure as naturally occurring antigen. Accordingly, said protein,

when produced by the method of the invention, is preferably produced in human cells.

Furthermore, the invention relates to an oligonucleotide hybridizing under stringent conditions to a portion of the nucleic acid molecular structure or the nucleic acid molecules of the invention, wherein said portion comprises said (missense) mutation or said stop codon or to the complementary portion thereof or hybridizing to a breakpoint of the gene conversion identified here in the above.

In this embodiment of the invention, it is understood that the oligonucleotides hybridizes directly to the mutated sequence or to the breakpoint. The setting of stringent hybridization conditions is well described, for example, in Sambrook et al, "Molecular Cloning, A Laboratory Handbook" CSH Press, Cold Spring Harbor 1989 or Hames and Higgins, "Nucleic acid hybridization, a practical approach", IRL Press, Oxford (1985). Thus, the detection of the specifically hybridizing sequences will usually require hybridization and washing conditions such as 0.2 x SSC, 0.1% SDS at 65°. As is well known, the length of the probe and the composition of the nucleic acid to be determined constitute further parameters of the stringent hybridization conditions. Preferably, the oligonucleotide is a deoxynucleotide. It is further preferred that the oligonucleotide comprises 12 to 50 nucleotides and more preferably 15 to 24 nucleotides. The hybridization to the breakpoint may be under stringent or non-stringent conditions. An example of non-stringent hybridization conditions is hybridization and washing at 50°C in 4 x SSC, 0,1% SDS.

Further, the invention relates to an antibody or an aptamer specifically binding to the protein product of the *RHD* gene of the invention.

The antibody may be tested and used in any serologic technique well known in the art, like agglutination techniques in tubes, gels, solid phase and capture techniques with or without secondary antibodies, or in flow cytometry with or without immunofluorescence enhancement.

The antibody of the invention may be a monoclonal antibody or an antibody derived from or comprised in a polyclonal antiserum. The term "antibody", as used in accordance with the present invention, further comprises fragments of said antibody such as Fab, $F(ab')_2$, Fv or scFv fragments; see, for example, Harlow and Lane, "Antibodies, A Laboratory Manual" CSH Press 1988, Cold Spring Harbor, N.Y. The antibody or the fragment thereof may be of natural origin or may be (semi) synthetically produced. Such synthetic products also comprise non-proteinaceous as semi-proteinaceous material that has the same or essentially the same binding specificity as the antibody of the invention. Such products may, for example, be obtained by peptidomimetics.

The term "aptamer" is well known in the art and defined, e.g., in Osborne et al., Curr. Opin. Chem. Biol. I (1997), 5-9 or in Stall and Szoka, Pharm. Res. 12 (1995), 465-483.

Furthermore, the invention relates to a method for testing for the presence of a nucleic acid molecule encoding a mutant Rhesus D antigen or of a nucleic acid molecule carrying a deletion of the *RHD* gene as characterized by the nucleic acid molecular structure or the nucleic acid molecule of the invention in a sample comprising hybridizing the oligonucleotide of the invention under stringent conditions to nucleic acid molecules comprised in the sample obtained from a human and detecting said hybridization.

Preferably, the method of the invention further comprises digesting the product of said hybridization with a restriction endonuclease or subjecting the product of said hybridization to digestion with a restriction endonuclease and analyzing the product of said digestion.

This preferred embodiment of the invention allows by convenient means, the differentiation between an effective hybridization and a non-effective hybridization. For example, if the wild type *RHD* gene comprises an endonuclease restriction site, the hybridized product will be cleavable by an appropriate restriction enzyme whereas a mutated sequence will yield no double-stranded product or will not

comprise the recognizable restriction site and, accordingly, will not be cleaved. Alternatively, the hybridizing oligonucleotide may only hybridize to the mutated sequence. In this case, only a hybrid comprising the mutated sequence, but not the wild type sequence, will be cleaved by the appropriate restriction enzyme. The analysis of the digestion product can be effected by conventional means, such as by gel electrophoresis which may be optionally combined by the staining of the nucleic acid with, for example, ethidium bromide. Combinations with further techniques such as Southern blotting are also envisaged.

Detection of said hybridization may be effected, for example, by an anti-DNA double-strand antibody or by employing a labeled oligonucleotide. Conveniently, the method of the invention is employed together with blotting techniques such as Southern or Northern blotting and related techniques. Labeling may be effected, for example, by standard protocols and includes labeling with radioactive markers, fluorescent, phosphorescent, chemiluminescent, enzymatic labels, etc.

The invention also relates to a method to simultaneously detect the presence of *RHDΨ* and any of the *RHD* molecular structures of the invention comprising hybridizing the oligonucleotide of the invention and at least an other oligonucleotide hybridizing to a *RHDΨ* structure under stringent conditions to nucleic acid molecules comprised in the sample obtained from a human and detecting said hybridization.

The present invention further relates to a method for testing simultaneously for the presence of *RHDΨ* and any of the *RHD* molecular structures of the present invention in a sample comprising determining the nucleic acid sequence of at least a portion of the nucleic acid molecular structure or nucleic acid molecule of the present invention, said portion encoding said (missense) mutation, said stop codon or a breakpoint of said hybrid gene and determining of at least a portion of a *RHDΨ* structure.

The invention additionally relates to a method for testing for the presence of a nucleic acid molecule encoding a mutant Rhesus D antigen or of a nucleic acid

molecule carrying a deletion of the *RHD* gene as characterized by the nucleic acid molecular structure or the nucleic acid molecule of the invention in a sample comprising determining the nucleic acid sequence of at least a portion of the nucleic acid molecular structure or the nucleic acid molecule of the invention, said portion encoding said (missense) mutation, said stop codon or a breakpoint of said hybrid gene.

Preferably, the method of the invention further comprises, prior to determining said nucleic acid sequence, amplification of at least said portion of said nucleic acid molecular structure.

Moreover, the invention relates to a method for testing simultaneously for the presence of *RHDΨ* and any of the *RHD* molecular structures of the present invention in a sample comprising carrying out an amplification reaction using a set of primers that amplifies at least a portion of said sequence wherein at least one of the primers employed in said amplification reaction is the oligonucleotide of the present invention and at least a primer amplifying (e.g. by specifically hybridizing to) a *RHDΨ* structure and analysing the amplified product(s).

RHDΨ is a *RHD* allele detected in D negatives that has been characterized by Singleton et al. (Singleton, B.K., Green, C.A., Avent, N.D., Martin, P.G., Smart, E., Daka, A., Narter-Olaga, E.G., Hawthorne, L.M., and Daniels, G. The presence of an RHD pseudogene containing a 37 base pair duplication and a nonsense mutation in africans with the Rh D- negative blood group phenotype. *Blood* 95(1):12-18, 2000).

Furthermore, the invention relates to a method for testing for the presence of a nucleic acid molecule encoding a mutant Rhesus D antigen or of a nucleic acid molecule carrying a deletion of the *RHD* gene as characterized by the nucleic acid molecular structure or the nucleic acid molecule of the invention in a sample comprising carrying out an amplification reaction using a set of primers that amplifies at least a portion of said sequence wherein at least one of the primers employed in said amplification reaction is the oligonucleotide of the invention.

Moreover, in a further embodiment the method of the invention wherein at least one of the primers employed in said amplification reaction is the oligonucleotide of the invention.

Preferably, amplification is effected by polymerase chain reaction (PCR). Other amplification methods such as ligase chain reaction may also be employed.

In a preferred embodiment of the method of the invention said PCR is PCR-RFLP, PCR-SSP or long-range PCR.

Additionally, in another preferred embodiment of the invention the molecular weight of the amplification product is analyzed. Said analysis of the molecular weight utilizes standard techniques, such as agarose gel electrophoresis, SDS-PAGE, mass spectrometry such as MALDI-TOF for this purpose, which are well known to the person skilled in the art.

In one preferred embodiment of the method of the invention, said method detects *RHD* positive alleles comprising the following steps:

(a) isolating DNA from a blood sample or blood donor;

(b) hybridizing at least two oppositely oriented primers under stringent conditions to the DNA so as to carry out a PCR;

(c) amplifying the target sequence;

(d) separating the amplification products on a gel; and

(e) analyzing the amplicons.

With regard to specific conditions to be applied in the various steps, it is referred to the corresponding description herein above.

In a preferred embodiment the *RHD* positive alleles are derived from a serologically RhD negative population. In another preferred embodiment the RhD-negative sample is selected from a Caucasian population.

The method of the invention will result in an amplification of only the target sequence, if said target sequence carries the or at least one mutation. This is because the oligonucleotide will, under preferably stringent hybridization conditions, not hybridize to the wild type sequence (with the consequence that no amplification product is obtained) but only to the mutated sequence. Naturally, primer oligonucleotides hybridizing to one or more as one, such as two mutated sequences may be employed in the method of the invention. The latter embodiment may be favorable in cases where combinations of mutations are tested for. It is important to note that not all or none of said mutations are necessarily missense mutations. This may be true for cases where other types of mutations occur in combination with the above missense mutations or with the above gene conversion.

Preferably, in the method of the invention said amplification or amplification reaction is or is effected by the polymerase chain reaction (PCR). Other amplification methods such as ligase chain reaction may also be employed.

Further, the invention relates to a method for testing for the presence of a protein product of the *RHD* gene of the invention in a sample comprising assaying a sample obtained from a human for specific binding to the antibody or aptamer or phage of the invention.

Testing for binding may, again, involve the employment of standard techniques such as ELISAs; see, for example, Harlow and Lane, "Antibodies, A Laboratory Manual" CSH Press 1988, Cold Spring Harbor.

In another preferred embodiment the invention relates to a method for testing for the presence of a protein product of the *RHD* gene encoding the nucleic acid molecular structure or the nucleic acid molecule of the invention, comprising utilizing direct agglutination methods, indirect antiglobulin tests, monoclonal anti-D antibodies and adsorption/elution techniques.

Thus, the embodiment may comprise direct agglutionation with two monoclonal anti-D antibodies, alternatively indirect antiglobulin tests using a gel matrix comprising an oligoclonal anti-D antibody, in a further alternative using monoclonal anti-Rhesus antibodies in another alternative adsorption of polyclonal anti-D antibodies to red cells and elution using a chloroform technique. Further description of the methods is given in example 18.

Preferably, in the method of the invention said sample is blood, serum, plasma, fetal tissue, saliva, urine, mucosal tissue, mucus, vaginal tissue, fetal tissue obtained from the vagina, skin, hair, hair follicle or another human tissue.

Furthermore, the method of the invention preferably comprises the step of enrichment of fetal cells. This enrichment may be achieved by using appropriate antibodies, lectins or other reagents specifically binding fetal cells or by any technique attempting the differential separation of maternal and fetal cells, like by density gradients. Also preferably, in said method fetal DNA or mRNA from maternal tissue like peripheral blood, serum or plasma may be extracted, advantageously according to conventional procedures.

In an additional preferred embodiment of the method of the invention, said nucleic acid molecule or proteinaceous material from said sample is fixed to a solid support.

Preferably, said solid support is a chip.

The advantages of chips are well known in the art and need not be discussed herein in detail. These include the small size as well as an easy access of computer based analysis of analytes.

Furthermore, the present invention relates to the use of the nucleic acid molecular structure or the nucleic acid molecule of the invention for the analysis of a negative or a positive Rhesus D phenotype.

The analysis can be effected, for example, on the basis of the methods described herein above.

The invention also relates to the use of the nucleic acid molecular structure or the nucleic acid molecule of the invention, the vector of the invention or the protein product of the *RHD* gene of the invention for the assessment of the affinity, avidity and/or reactivity of monoclonal anti-D or anti-C antibodies or of polyclonal anti-D or anti-C antisera or of anti-globulin or of anti-human-globulin antisera or of preparations thereof.

Anti-C is a monoclonal antibody or polyclonal antiserum binding to antigen C.

The invention also relates to the use of cells, preferably red blood cells, from probands carrying the nucleic acid molecular structure or the nucleic acid molecule of the invention for the assessment of the affinity, avidity and/or reactivity of monoclonal anti-D or anti-C antibodies or of polyclonal anti-D or anti-C antisera or of anti-globulin or of anti-human-globulin antisera or of preparations thereof.

Said preparations can be provided according to techniques well known in the art. Said preparations may comprise stabilisators such as albumins, further sodium azide, salt ions, buffers etc. The formulation of the preparation may have an influence on the binding characteristics of the antibodies, as is well known in the art.

For example, in a first step, the Rhesus D gene of a carrier or of a blood donor and its allelic status is analyzed and it is determined whether said gene comprises a mutation that was found in accordance with the present invention. In a second step, said mutation is correlated to a certain RhD antigen density on the surface of red blood cells. Conveniently, said correlation can be established by data provided in the present invention (such as mutations per se) and techniques that are well known in the art (see, e.g. Jones et al. 1996, Flegel and Wagner, 1996). In a third step, the features of an antibody or an antiserum such as reactivity, sensitivity, affinity, avidity, and/or specificity are determined with suitable blood group

serological techniques preferably using red blood cells that were molecularly and with respect to the RhD antigen surface density characterized as described in step 2. Such data can be used, for example, in quality controls, standardization, etc.

The invention will be most useful for the characterization, standardization and quality control of monoclonal and polyclonal antisera, preferably anti-D monoclonals or antisera. Further, for example, anti-globulin and anti-human-globulin antisera can be characterized on the basis of the teachings of the present invention. An appropriately characterized anti-D monoclonal antibody can be conveniently used in RhD diagnostics. For example, a suitably characterized monoclonal antibody will be useful in determining the D antigen density on the surface of blood cells. Cut-off values for monoclonal antibodies useful in diagnosis can thus be established. This is important for the quality control of antibodies used in RhD diagnosis.

Thus, the invention also relates to a method for the characterization of monoclonal antibodies or polyclonal antisera or of a preparation thereof, said method comprising

(a) testing the nucleic acid of sample of a proband for the presence of a breakpoint or mutation as defined in accordance with the invention;

(b) correlating, on the basis of the mutation or deletion status and the allelic status of the *RHD* gene, the nucleic acid with the density of the protein product of the *RHD* gene on the surface of red blood cells of said proband;

(c) reacting said monoclonal antibodies or polyclonal antisera or said preparation thereof with a cell carrying the protein product of the *RHD* gene on its surface;

(d) characterizing said monoclonal antibodies or polyclonal antisera or said preparation thereof on the basis of the results obtained in step (c).

As regards the term "allelic status", this term describes the possibilities that the *RHD* alleles in a proband are present in a homozygous, heterozygous or hemizygous state. Also comprised by this term is the possibility that the two alleles carry two different mutations (including the conversion) defined herein above.

In a preferred embodiment of the method of the invention, said characterization comprises the determination of reactivity, sensitivity, avidity, affinity, specificity and/or other characteristics of antibodies and antisera.

Furthermore preferred is a method wherein said cell carrying the protein product of the *RHD* gene on its surface is a red blood cell.

The invention also relates to a method for determining whether a patient in need of a blood transfusion is to be transfused with RhD negative blood from a donor comprising the step of testing a sample from said patient for the presence of one or more nucleic acid molecular structures or nucleic acid molecules of the invention, wherein a positive testing for two different of said nucleic acid molecular structures or nucleic acid molecules is indicative of the need for a transfusion with Rh negative blood. Alternatively, a positive testing indicating the concomitant presence of two identical copies of one of said nucleic acid molecular structures or nucleic acid molecules is indicative of the need for a transfusion with Rh negative blood.

Alternatively, a negative testing for the presence of the nucleic acid molecular structure or nucleic acid molecule representative of the common *RHD* negative haplotype with or without a negative testing for one or more nucleic acid molecular structures or nucleic acid molecules representative of the other *RHD* negative nucleic acid molecular structures or nucleic acid molecules of this invention permits the transfusion of blood that is typed as RhD positive. The invention has important implications for devising a transfusion therapy in humans. For example, it can now be conveniently tested whether the patient actually needs a transfusion with a RhD negative blood or whether such precautions need not be taken.

The invention also relates to a method for determining whether blood of a donor is suitable for transfusion to a patient in need thereof who should not be exposed to antigen C comprising the step of testing a sample from said donor for the presence of the nucleic acid molecular structure of the present invention wherein a positive

testing for the nucleic acid molecular structure of the present invention precludes the transfusion of the donor's blood.

Furthermore, the invention relates to a method for determining whether blood of a donor may be used for transfusion to a patient in need thereof comprising the step of testing a sample from said donor for the presence of one or more of said nucleic acid molecular structures or nucleic acid molecules of the invention, wherein a negative testing for the nucleic acid molecular structures representative of the common *RHD* negative haplotype with or without a negative testing for one or more nucleic acid molecular structures or nucleic acid molecules representative of the other *RHD* negative haplotypes of this invention excludes the transfusion the donor's blood to a patient that is typed as RhD negative.

The invention also relates to a method for determining whether the blood of a donor may be transfused to a patient typed as RhD negative comprising the step of testing a sample from said donor for the presence of one or more nucleic acid molecular structures or nucleic acid molecules of the invention, wherein a positive testing for two different of said nucleic acid molecular structures or nucleic acid molecules is indicative of the possibility to transfuse the donor's blood to a patient typed as RhD negative.

Alternatively, a positive testing indicating the concomitant presence of two identical copies of one of said nucleic acid molecular structures or nucleic acid molecules is indicative of the possibility to transfuse this donor's blood to a patient that is typed as RhD negative.

The samples referred to in the above recited methods may be samples that are referred to throughout the specification, such as blood, serum, etc.

As regards the guidelines for transfusing a patient on the basis of any of the above recited methods, the utmost care must be taken that suboptimal transfusion policy is avoided. The risk factor is always to be considered by the physician in charge. In all cases, the potential risk for the patient is to be minimized.

The invention also relates to a method for assessing of the risk of a RhD negative mother of conceiving or carrying an RhD positive fetus or of the risk of a mother having an anti-D titer of conceiving or carrying a fetus at risk to develop hemolytic disease of the newborn comprising assessing a sample obtained from the father of the fetus for the presence of one or more of said nucleic acid molecular structures or nucleic acid molecules of the invention, wherein a negative testing for nucleic acid molecular structures or nucleic acid molecules representative of the common *RHD* negative haplotype with or without a negative testing for one or more nucleic acid molecular structures or nucleic acid molecules representative of the other *RHD* negative haplotypes of this invention is indicative for a high risk of conceiving an RhD positive fetus.

In a preferred embodiment of the method of the present invention said nucleic acid molecular structure carries mutations or deletions.

The invention also relates to a method for determining whether the father is RhD negative comprising the step of testing a sample from the father for the presence of one or more nucleic acid molecular structures or nucleic acid molecules of the invention, wherein a positive testing for two different of said nucleic acid molecular structures or nucleic acid molecules is indicative of the father being RhD negative.

Alternatively, a positive testing indicating the concomitant presence of two identical copies of one of said nucleic acid molecular structures or nucleic acid molecules representative of *RHD* negative haplotypes is indicative of the father being RhD negative.

Furthermore, the invention relates to a method for assessing the possibility or likelihood of a man being the father of a child by assaying a sample obtained from said man for the presence of one or more of said nucleic acid molecular structures or nucleic acid molecules of the invention, wherein the test results are used to determine the homozygosity for, the heterozygosity for or the absence of any nucleic acid molecular structures or nucleic acid molecules representative of the

RHD negative haplotype of the present invention used to infer the possibility or likelihood of said man being the father of the child.

The preparation may be a diagnostic or pharmaceutical preparation.

The pharmaceutical composition of the present invention may further comprise a pharmaceutically acceptable carrier and/or diluent. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Compositions comprising such carriers can be formulated by well known conventional methods. These pharmaceutical compositions can be administered to the subject at a suitable dose.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like. Furthermore, the pharmaceutical composition of the invention may comprise further agents such as interleukins or interferons depending on the intended use of the pharmaceutical composition.

The invention also relates to a method of treating a pregnant woman being Rhesus D negative wherein the fetus does not carry two nucleic acid molecular structures or nucleic acid molecules of the invention or is not homozygous for any nucleic acid molecular structure or nucleic acid molecule of the invention, comprising administering anti-D to said woman.

Pregnant women may be currently treated with an anti-D prophylaxis, when a Rhesus negative mother carries a RhD positive fetus. The invention allows the discrimination of an anti-D prophylaxis requirement depending on the status of the mother's and/or the fetus' possessing a RhD protein of the invention. One or more of the RhD proteins of the invention may be prone to immunization of their carriers and, hence, would be indicative for the therapy of the mother. Similarly, one or more RhD proteins of the invention, when carried by the fetus, may be known to be of low immunogenicity to the mother and, hence, would be indicative for the omission of anti-D prophylaxis in difference to current clinical therapy.

The administration can be effected by standard routes and doses which can be defined by the attending physician; Mollison, 1993. Preferably, a monoclonal anti-D or combinations/mixtures of monoclonal anti-Ds is/are administered in doses of 50 µg to or exceeding 500 µg anti-D antibody/antisera for intravenous or intramuscular administration (Bowman, 1998). For the quality control of these anti-D antibodies/antisera, the results and methods provided by the present invention may be advantageously employed.

The invention also relates to the use of a phage, aptamer, monoclonal antibody or a polyclonal antisera or a preparation thereof as characterized in the present invention for determination of the protein product of the *RHD* gene.

In a preferred embodiment of said use, said determination of the protein product of the *RHD* gene is effected in connection with blood group typing.

Furthermore, the invention relates to a preparation comprising the antibody or aptamer or phage of the invention.

The present invention also relates to a method of identifying an antibody V_H or V_L chain or a combination thereof or an aptamer specifically binding to a protein product of the *RHD* gene of the invention comprising

(a) contacting the protein product of the *RHD* gene of the invention with a phage library displaying V_H or V_L chains or combinations thereof on the surface of the phage or with aptamers;

(b) identifying phage or aptamers that bind to said protein product of the *RHD* gene; and optionally

(c) repeating steps (a) and (b) one or more times.

The preparation of phage library and the screening/identification of desired antibody (chains) per se is well known in the art and reviewed, for example, in Winter et al., Annu. Rev. Immunol. 12 (1994), 433-455 and references cited therein. Also, aptamers can be prepared and cloned in phage according to conventional protocols. Whereas single V_H or V_L chains may be identified by the method of the invention as binding to the protein product of the *RHD* gene of the invention, it is preferred to identify V_H-V_L combinations expressed by the phage because this situation resembles the situation of natural antibody binding. By repeating steps (a) and (b) one or more times, better binding specificities may be identified. Protocols for the optimization of binding properties such as affinities, including elution steps for removing bound phage, are well established in the art. For example, once a V_H chain with a convenient binding capacity has been found, V_L chains may be identified that significantly improve the binding capacity of the antibody, e.g. by replacing the V_L chain that was associated with the V_H chain in the first selection step with a more suitable V_L chain.

The invention also relates to a method of identifying a monoclonal antibody specifically binding to a protein product of the *RHD* gene of the invention comprising

(a) contacting the protein product of the *RHD* gene of the invention with one or more monoclonal antibodies;

(b) identifying monoclonal antibodies that bind to said protein product of the *RHD* gene; and optionally

(c) repeating steps (a) and (b) one or more times.

The invention also relates to a method of identifying an antibody V_H or V_L chain or a combination thereof or an aptamer specifically binding to a protein product of the *RHD* gene of the invention comprising

(aa) contacting said protein product of the *RHD* gene and

(ab) a normal D polypeptide

wherein the normal D polypeptide is present in a molar mass that is higher, equal or less than the protein product of the *RHD* gene of (aa) with a phage library displaying V_H or V_L chains or combinations thereof on the surface of the phage or with aptamers;

(b) identifying phage or aptamers that bind to said protein product of the *RHD* gene of (a); and optionally

(c) repeating steps (a) and (b) one or more times.

Particularly preferred in step (ab) is that the molar mass of the normal D polypeptide is higher than that of the protein product of the *RHD* gene of (aa).

In the case that only one round of selection is employed for the identification (i.e. when step (c) does not apply), it is preferred that the number of protein product of the *RHD* gene of (aa) is in molar excess over the number of phage particles. The preferred embodiments of the method of identifying an antibody V_H or V_L chain or of a combination thereof or of an aptamer described hereinbefore equally apply to this embodiment of the invention.

The invention also relates to a method of identifying a monoclonal antibody specifically binding to a protein product of the *RHD* gene of the invention comprising

(aa) contacting the protein product of the *RHD* gene and

(ab) a normal D polypeptide

wherein the normal D polypeptide is present in a molar mass that is higher, equal or less than the protein product of the *RHD* gene of (aa) with one or more monoclonal antibodies;

(b) identifying monoclonal antibodies that bind to said protein product of the *RHD* gene of (aa); and optionally

(c) repeating steps (a) and (b) one or more times.

Preferably, the protein product of the *RHD* gene is exposed on the surface of a cell. An appropriate surface is the surface of an erythrocyte. However, other host cells may be transfected with a vector suitable for expression of the protein product of the *RHD* gene of the invention and express the same on their surface. Antibodies may also bind to recombinant proteins of or parts of proteins of D antigen and purified proteins.

It is further preferred that the polypeptide or host cell is affixed to a solid support. Suitable examples for solid supports are microtiter plates or beads.

In an additionally preferred antibody, subsequent to step (b) or (c), the following step is carried out:

(d) identifying the amino acid sequence of the V_H or V_L chains and/or identifying the nucleic acid sequences encoding said amino acid sequence.

The identification of the amino acid/nucleic acid sequences can be effected according to conventional protocols; see, e.g., Sambrook et al., loc. cit.

Hence and in summary, the present invention provides means and methods for the detection of *RHD* haplotypes, comprising common *RHD* negative haplotypes, as described above, as well as presumably rare *RHD* positive allels in serologically RhD negative populations. Latter alleles, harbouring *RHD* sequences and therefore determined as *RHD*-positive, can comprise either *RHD/RHCE* hybrid genes, stop codons, splice site mutations or gene deletions, that terminate or reduce the RhD antigen expression. Carrying out the improved detection methods of the invention, it was surprisingly found, that several samples, determined as RhD negative in routine serology, could be identified having *RHD* positive alleles. Furthermore, some of those samples were even RhD antigen positive when performing a detection assay based on adsorbtion and elution, indicating that the molecular basis for the *RHD* positive alleles in RhD negatives is more heterogenous than anticipated. Advantageously, the disclosure content of the present invention now

provides new and practicable nucleic acid amplification techniques to determine whether *RHD* specific sequences cause RhD positive or RhD negative phenotypes.

In a particularly preferred embodiment the method of present invention, wherein, in the case that only one round of selection is employed for the identification, the number of protein molecules of the *RHD* gene of (a) is in molar excess over the number of phage particles.

Moreover, the present invention relates to the use of cells, preferably red blood cells comprising the protein product of the *RHD* gene of the present invention or produced by the method of the present invention, from probands for the assessment of the affinity, avidity and/or reactivity of monoclonal anti-D or anti-C antibodies of the present invention or of polyclonal anti-D or anti-C antisera or of anti-globulin or of anti-human-globulin antisera or of preparations thereof.

Furthermore, the invention relates to the use of *SMP1* polymorphisms to determine specific *RH (RHD-RHCE)*-haplotypes genetically linked to said polymorphisms.

Basis for this embodiment of the present invention is provided by the unique structure of the *RH* locus comprising three genes: *RHD*, *RHCE,* and *SMP1.* The nucleotide sequence of the latter gene has been deposited in the Genbank as putative member of an 18 kDa small membrane protein family. Its function is as yet unknown. It shows homology to an open reading frame on chromosome 21 (Reboul, Genome res. 9:242, 1999). Its position between both *RH* genes implies that any polymorphism of the *SMP1* gene would be tightly linked to a specific *RH* haplotype, and it might be anticipated that functionally relevant mutations of the *SMP1* gene may cause selection pressure for or against specific *RH* haplotypes. Such factors might explain some previously unresolved issues of *RH* haplotype distribution, like the high frequency of *RH* negatives in Europe. Screening for polymorphisms in *SMP1* is therefore of high interest for the understanding of the *RH* locus as well as for diagnostic applications thereof.

According to the present invention the term "polymorphism" relates to the existence in a population of more than one genetic structure or a gene of a haplotype or of a DNA segment. Nevertheless, sometimes such a genetic polymorphism does not always result in a differing phenotype, but may only be detected at the genetic level.

In another preferred embodiment the invention relates to a method to detect specific *RH* (*RHD-RHCE*)-haplotypes comprising the determination of *SMP1*-polymorphisms in the *SPM1* gene by utilizing any structural feature or nucleotide sequence or both of the *RH* locus or combinations thereof with techniques known in the art, preferably by PCR-RFLP, PCR-SSP or long-range PCR.

Furthermore, the invention relates to a kit comprising

(a) the oligonucleotide of the invention; and/or
(b) the antibody of the invention;
(c) the aptamer of the invention; and/or
(d) the phage of the invention;
(e) a pair of primers useful for carrying out the amplification reaction of the invention.

Parts of the kit can be packaged individually in vials or in combination in containers or multicontainer units. The kit of the present invention may be advantageously used for carrying out the method of the invention and could be, inter alia, employed in a variety of applications referred to above. The manufacture of the kits follows preferably standard procedures which are known to people skilled in the art.

The present invention also relates to a process to determine the presence of an antigen C encoded by a *RHD* gene comprising the step of detecting any of the breakpoint regions mentioned in the present invention.

Finally, the invention relates to a process to determine the presence of an antigen C comprising the steps of the processes of the present invention.

The disclosure content of the documents as cited in this specification is herewith incorporated by reference.

The figures show

Figure 1 Schematic structure of the *RH* gene locus. The positions and orientations of the genes and the *Rhesus boxes* are indicated by open arrows and triangles, respectively (Panel A). The exons are shown as vertical bars and their exon number is indicated. The two *RH* genes have opposite orientation, face each other with their 3' ends, and are separated by about 30,000 bp. A third gene, *SMP1*, has the same orientation as *RHD* and is positioned in between *RHD* and *RHCE*. The *RHD* gene is flanked on both sides by the two highly homologous *Rhesus boxes* (b). All exons are shorter than 200 bp with the exception of the *RHD* and *SMP1* 3' terminal exons. Data used to establish this structure (Panel B) include the extension of genomic sequences represented in the cDNAs (horizontal arrows), identities and homologies to genomic clones (bar a: identity with dJ465N24; b: homology of *RHD* to dJ469D22; c: homology of *RHD* 3' part to dJ465N24; d: identity with dJ469D22). The positions of three bridging PCR reactions are indicated. The correct position of a nucleotide stretch previously reported by Okuda et al. (Okuda, Biochem. Biophys. Res. Commun. 263:378, 1999) as "spacer" sequence between *RHD* and *RHCE* is indicated by the bar labeled s.

Figure 2 Chromosomal organization of the DNA regions located 5' to the *RHD* and *RHCE* genes. The proposed structure of the *RHCE* and *RHD* 5' flanking regions is depicted (Panel A). A total of 4,941 bp immediately 5' of the ATG start codons are homologous between the *RHCE* and *RHD* genes (vertically hatched bars). No homology is present further beyond this homology region (diagonally hatched bars). Two genomic clones, dJ469D22 and dJ465N24, were utilized for primer design. DJ469D22 comprises the full length of the depicted *RHCE* region,

whereas dJ465N24 extends only 466 bp into the homology region. The positions of several PCR primers are indicated (a, rey14a; b, rend32; c, rey15a; d, re014; e, re011d). This proposed structure is supported by several PCR reactions (panel B). Forward priming was done with primer a (*RHCE* specific, lane 1 - 3), primer b (*RHD* specific, lane 4 - 6), and primer c (*RHCE* and *RHD* homology region, lane 7 - 9). Amplicons were lacking for primer a with *RHD* specific reverse primer e (lane 2) and for primer b with *RHD* negative DNA (lane 6). The other seven PCR reactions yielded amplicons of the predicted sizes in accordance with the genomic structure shown in panel A.

Figure 3 Chromosomal organization of the *SMP1* gene. The *SMP1* gene has seven exons. The positions and approximate sizes of the introns are shown. The start of the published cDNA (GenBank accession number AF081282) is separated by 15 nucleotides from the downstream *Rhesus box*. Exon 1 contains only 5' untranslated sequence, the *SMP1* start codon is located in exon 2. Exon 7 contains 16 codons and 1,656 bp 3' untranslated sequences and is contiguous with the 3' untranslated sequence of *RHCE* exon 10.

Figure 4 Chromosomal organization of the *Rhesus boxes*. The physical extension of the upstream *Rhesus box* (5' to *RHD*) is 9,145 bp (black bar). About 63% of the boxes' nucleotide sequence consists of repetitive DNA; the types of the repeat families are indicated. The overall homology between the upstream and downstream *Rhesus box* is 98.6%, but within an 1,463 bp identity region (horizontal arrows), there is only a single 4 bp insertion (double vertical line). A CpG-island (double-headed arrow) is located at the 3' end and is in the downstream *Rhesus box* (3' to *RHD*) adjacent to the *SMP1* promoter.

Figure 5 *RHD* gene deletion in the Rh negative haplotypes. Three 3,100 bp segments of the *Rhesus boxes* are shown. The upper line indicates the nucleotide sequence of the upstream *Rhesus box* in D-positives, the lower line the nucleotide sequence of the downstream *Rhesus box* in D-positives. The middle line gives the nucleotide sequence of the single *Rhesus box* carried by Rh negatives. Asterisks denote identical nucleotides. The *RHD* deletion occurred in a 903 bp segment of absolute identity that was part of a 1,463 bp identity region. The positions of primers rez7 and rnb31 is shown (m indicates mismatch). *Pst*I restriction sites are indicated by carets (^). The three *Rhesus boxes* are deposited at EMBL under accession numbers AJ252311 (upstream *Rhesus box*), AJ252312 (downstream *Rhesus box*), and AJ252313 (hybrid *Rhesus box*).

Figure 6 Two technical procedures for specific detection of the *RHD* deletion in the common *RHD* negative haplotypes. A long-range PCR amplification with primers located in non-*Rhesus box* sequences (Panel A) and PCR-RFLP with primers located in the *Rhesus boxes* are shown (Panel B). The deduced genotypes are indicated. The primers of the long-range PCR were located 5' of the upstream *Rhesus box* (primer rez4) and in *SMP1* exon 1 (primer sr9). *RHD* negative haplotypes were detected specifically (Panel A, lane 1 - 6). DNA homozygous for the *RHD* gene was negative, because the PCR cannot amplify the 70,000 bp DNA stretch of the *RHD* gene. For the PCR-RFLP method, the PCR amplicons (primer rez7 and rnb31) were digested with *Pst*I. In D-negatives, there are three *Pst*I sites in the amplicon (see Fig. 5) resulting in fragments of 1,888 bp, 564 bp, 397 bp, and 179 bp (lane 1 to 3). The downstream *Rhesus box* of D-positives lacks one *Pst*I-site resulting in fragments of 1,888 bp, 744 bp, and 397 bp (lane 7 to 9). RHD^+/RHD^- heterozygotes show both fragments of 744 and 564 bp (lane 4 to 6). The 564 bp fragment appears weaker because heterodimers are not cut by *Pst*I. Primer rnb31 does not amplify the upstream *Rhesus box* of D-positives.

Figure 7 Model of the proposed mechanism causing the prevalent *RHD* negative haplotypes in whites. The physical structure of the *RHD* and *RHCE* gene locus is depicted (panel A). An unequal crossing over between the upstream and downstream *Rhesus boxes* can be triggered by their high homology (panel B). The breakpoint region in the *Rhesus boxes* was found to be of 100% homology for 903 bp (see Fig. 5). Resolving the crossed over chromosome yields the *RH* gene structure of the extant *RHD* negative haplotype (panel C).

Figure 8 DNA sequence of the hybrid *Rhesus box* of *RHD* negatives.

Figure 9 DNA sequence of the upstream *Rhesus box* of D-positives.

Figure 10 DNA sequence of the downstream *Rhesus box* of D-positives.

Figure 11 DNA sequence of the *RHD* promoter. The last three nucleotides represent codon 1 of the *RHD* gene.

Figure 12. Cde^s breakpoint region in *RHD* intron 3. The nucleotide sequence of a part of the intron 3 of Cde^s, *RHD* and *RHCE* 2,938 to 3,636 bp 3' of the exon 3/intron 3 junction is shown. The human DNA sequence from clone RP3-469D22 on chromosome 1p35.1-36.13 containing the 5' part of the gene for *RHCE* (GenBank accession number AL031284) was taken as reference; numbers indicate the position in this sequence relative to the first base of intron 3 in the *RHCE* gene. The corresponding *RHD* gene sequence derives from GenBank accession number AL139426. Nucleotides indicating *RHD* or *RHCE* origin of the Cde^s sequences are highlighted. A 154 bp DNA stretch comprising the breakpoint region of Cde^s is indicated by asterisks.

Figure 13. *Cde*^{s} breakpoint region in *RHD* intron 7. The nucleotide sequence of a part of the intron 7 of *Cde*^{s}, *RHD* and *RHCE* about 2,726 to 3,719 3' of the exon 7/intron 7 junction is shown. The human DNA sequence from clone RP3-469D22 on chromosome 1p35.1-36.13 containing the 5' part of the gene for *RHCE* (GenBank accession number AL031284) was taken as reference; numbers indicate the position in this sequence relative to the first base of intron 7 in the *RHCE* gene. The corresponding *RHD* gene sequence derives from GenBank accession number AL139426. Nucleotides indicating *RHD* or *RHCE* origin of the *Cde*^{s} sequences are highlighted. A 666 bp DNA stretch comprising the breakpoint region of *Cde*^{s} is indicated by asterisks.

Figure 14. Specific detection of *Cde*^{s} by PCR-SSP. A PCR-SSP detecting the 3' breakpoint region of *Cde*^{s} in intron 7 is shown. Both, a *RHD* negative sample (lane 1, ccddee) and a normal *RHD* positive sample (lane 2, ccD.EE) yield the 434 bp control product only, which is derived from the *HGH* gene. In contrast, a *Cde*^{s} sample (CcddEe, lane 3) yields the 338 bp specific product, which is derived from the breakpoint region in intron 7, and in addition the 434 bp control fragment. This reaction is specific for *Cde*^{s}; the two partial D phenotypes D^{IVa} (lane 4) and D^{III} type IV (lane 5) do not yield a specific product. The reaction also detects *Cde*^{s} specifically, if *Cde*^{s} occurs *in trans* to other *RHD* alleles, like in a *RHDΨ/Cde*^{s} sample (lane 6).

Figure 15. *RHD* PCR-SSP for routine DNA typing. The PCR is performed as a modular system consisting of two multiplex reactions, an intron 4/exon 7 multiplex PCR-SSP (Panel A) and an intron 7 PCR enhanced by specific detection of *RHD*(W16X) and *RHDΨ* (Panel B). Results are shown for a normal D positive sample (lane 1), a normal D negative sample (lane 2), several rare D negative samples (lanes 3 to 6) and major D positive *RHD* variants (lanes 7 and 8). Standard D positive and D negative samples and D categories IV and VI are recognized in reaction A. *RHD-CE(8-9)-D* is detected in reaction B by the absence of the intron 7 band. The presence of *RHD*(W16X) and *RHDΨ* is also detected in reaction B. Band size is Panel A, control, 434 bp (HGH gene); intron 4, 226 bp; exon 7, 123 bp; Panel B, control, 659 bp (chromosome 1 genomic sequence about 90,000 bp 5' of *Rhesus box*); intron 7, 390 bp; *RHD*(W16X), 248 bp; *RHDΨ*, 154 bp. The internal control amplicons, which were devised to be larger than the specific amplicons, may be suppressed because of competition, if a specific product is amplified.

The examples illustrate the invention:

Example 1: Blood samples and DNA isolation

EDTA- or citrate-anticoagulated blood samples were collected from white blood donors and characterized as D negative in routine typing including an antiglobulin test with anti-D (Wissenschaftlicher Beirat der Bundesärztekammer; Paul-Ehrlich-Institut. Richtlinien zur Blutgruppenbestimmung und Bluttransfusion

(Hämotherapie). Köln: Deutscher Ärzte-Verlag; 1996; Wagner, Infusionsther Transfusionsmed 22:285-90, 1995). If necessary, samples were collected at random for specific CcEe phenotypes. A total of 314 ccddee, 433 Ccddee, 271 ccddEe, 19 CcddEe, 24 CCddee, 1 CcddEE and 6 ccddEE samples were tested. DNA was isolated by a modified salting-out procedure as described in Gassner et al., Transfusion 37; 1020, 1997.

Example 2: Molecular work-up

All samples were tested by PCR-SSP for the presence of four different *RHD* specific polymorphisms located in the *RHD* promoter, intron 4, exon 7 and the 3' untranslated region of exon 10. 48 samples with at least one positive PCR reaction were detected (Table 5). Those samples were further investigated for the presence of *RHD* specific polymorphisms in exon 3, exon 4, exon 5, exon 6, exon 7, intron 7 and exon 9. Twenty-six samples showed one of eight distinct PCR patterns involving a mixture of positive and negative reactions (Table 6). Twenty-two samples were positive for all *RHD* specific polymorphisms investigated and were assigned to eight *RHD* alleles by *RHD* specific sequencing of the ten *RHD* exons from genomic DNA (Table 7). For each PCR pattern and each *RHD* allele, one sample was serologically investigated. The phenotypes were determined to represent weak D, partial D, and D_{el}, or confirmed as serologically D negative by adsorption/elution (Table 6 and 7).

Example 3: DNA database searches and analysis

The GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) and the chromosome 1 database of the Sanger Center (http://www.sanger.ac.uk/cgi-bin/nph-Blast_Server.html) were searched with cDNA sequences representative of *RHD* (RhXIII, accession number X63097) and *RHCE* (RhVI, X63095) using the BLAST program. The 84,810 bp genomic clone dJ469D22 (GenBank accession number AL031284), the 129,747 bp genomic clone dJ465N24 (GenBank accession number AL031432) and the 2,234 bp *SMP1* cDNA (GenBank accession number AF081282) were identified. dJ469D22

represented a major fragment of the *RHCE* gene, starting 33,340 bp 5' of the *RHCE* start codon and ending 1,142 bp 3' of exon 9. In dJ465N24, an internal stretch of 1,418 bp located between position 120,158 and 121,568 was 96% homologous to the 3' end of the *RHD* cDNA. The 3' end of the *SMP1* cDNA was complementary to the 3' end of the *RHCE* cDNA with an overlap of 58 bp.

Example 4: PCR

If not mentioned otherwise, PCR reactions were done with 60°C annealing, 10 min extension at 68°C and denaturation at 92°C using the expand long template or the expand high fidelity PCR systems (Boehringer Mannheim, Mannheim, Germany) and the listed primers (Table 1). Three PCR reactions were used to bridge gaps in the 3' flanking regions of the *RH* genes. PCR 1 was done using primers rea7 and rend31 (PCR 2, rend32, sf1c; PCR 3 , rea7, sf3). The structure of the 5' flanking regions was confirmed with PCR amplifications involving sense primers rend32, rey14a, rey15a and antisense primers re011d and re014. Intron 9 size was estimated to be about 9,000 bp based on PCR amplifications using rb10b and rr4 for *RHD* (re96 and rh7 for *RHCE*).

Example 5: Nucleotide sequencing

Nucleotide sequencing was performed with a DNA sequencing unit (Prism BigDye terminator cycle-sequencing ready reaction kit; ABI 373A, Applied Biosystems, Weiterstadt, Germany).

Example 6: Characterizing the *RH* gene locus

A physical structure of the *RH* genes' locus was derived (Fig. 1). This structure was deduced from the following considerations: **(i) 3' flanking regions.** The 3' flanking region of *RHD* was highly homologous to the 3' part of dJ465N24 (Fig. 1B, region c). This homology continued beyond the end of the *RHD* cDNA and extended for at least 8,000 bp as proven by the fact that it was possible to obtain PCR amplicons (Fig. 1B, PCR 1). Sequences homologous to the 3' part of dJ465N24 were

neighboring to the 5' region of the *SMP1* gene (Fig. 1B; PCR 2). The 3' end of the *SMP1* gene occurred immediately adjacent to the *RHCE* gene as indicated by the complementarity of the 3' ends of the respective cDNAs and confirmed by PCR (Fig. 1B, PCR 3). Further details of the *RHD* 3' flanking region (Rhesus *box*) and the *SMP1* gene are described below. **(ii) 5' flanking regions**. dJ469D22 comprised 33,340 bp 5' flanking region of *RHCE*. For *RHD*, a 466 bp homology between the 3' end of dJ465N24 and dJ469D22 indicated that dJ465N24 might represent the 5' flanking sequence of *RHD*. This assumption was proven by PCR (Fig. 2). **(iii) Analysis of YAC 38A-A10**. DNA from the YAC 38A-A10 (UK HGMP resource centre, Cambridge, UK) was isolated after a single growth phase by standard methods (http://hdklab.wustl.edu/lab_manual/yeast). It was confirmed that this YAC contained *RH* DNA. Furthermore, shotgun cloning experiments indicated that some of its insert probably derived from the X chromosome (data not shown). This YAC had been known to contain *RHCE* exons 2 to 10 and *RHD* exons 1 to 10 (Carritt, Hum. Mol. Genet. 6:843, 1997) and was thus expected to contain the DNA segments interspersed between *RHD* and *RHCE*. The presence of DNA segments representative of different parts of the *RH* locus in this YAC was observed (Table 2). The results were concordant with the proposed structure of the *RH* locus shown in Fig. 1, Panel A.

Example 7: Identification of *RHD* specific sequences in the *RHD* promoter

About 2,000 bp *RHD* promoter sequence was established by chromosomal walking (GenomeWalker kit, Clontech, Heidelberg, Germany). D-positive and D-negative samples were amplified using primers re04 and re11d (Table 1) and *RHD*- and *RHCE*-specific sequences established for 1,200 bp 5' of the start codon by sequencing with internal primers. A short deletion in the *RHD* gene was identified and used to develop the *RHD*-specific primer re011d. The 1,200 bp sequence including the *RHD* promoter has been deposited at EMBL under accession no. AJ252314.

Example 8: Characterization of *Rhesus boxes*

Two DNA segments of about 9,000 bp, located 5' and 3' of the *RHD* gene, were highly homologous, had identical orientation, and were designated "*Rhesus boxes*" (Fig. 4). The *Rhesus boxes* were amplified and sequenced using internal primers in two overlapping fragments using PCR primer pairs rez4/rend31 and rend32/re011d (upstream *Rhesus box*), rea7/rend31 and rend32/sr9 (downstream *Rhesus box*), and rez4/rend31 and rend32/sr9 (hybrid *Rhesus box* of *RHD*-negative). The upstream *Rhesus box* (5' of *RHD*) was about 9,142 bp long and ended about 4,900 bp 5' of the *RHD* start codon. The downstream *Rhesus box* (3' of *RHD*) was 9,145 bp long and started 104 bp after the *RHD* stop codon. The *Rhesus boxes* exactly embraced the part of *RHD* with homology to *RHCE*. The central portion of both *Rhesus boxes* contained an almost complete remnant of a transposon-like human element (THE-1B). The single open reading frame usually found in the THE-1B element was, however, abolished due to several nucleotide aberrations occurring in both *Rhesus boxes* in parallel, including a nonsense mutation in codon 4. While there was overall 98.6% homology between both *Rhesus boxes*, a 1,463 bp "identity region" located between positions 5,701 and 7,163 was completely identical with the single exception of a 4 bp T insertion in a poly T tract.

Example 9: Evaluation of the genomic structure of *SMP1*

The genomic structure of the *SMP1* gene was evaluated by PCR using internal primers and nucleotide sequencing (Fig. 3). The sizes of the *SMP1* introns were estimated by PCR amplicons obtained with primers rend32, sr9, sf1c, sf1, sm19, sr45, sr47, sr47c, sr5, sr5c, sr55, sr55c, sr3, sr3kp, rea7. The positions of the intron/exon junctions and the absence of additional introns were determined by nucleotide sequencing. Six introns could be identified. Exon 1 contained 5' untranslated sequences only and was separated from the Rhesus *box* by 15 bp. The long 3' untranslated sequence of exon 7 overlapped with *RHCE* exon 10. The total gene size was estimated to be 20,000 bp resulting, in conjunction with the

downstream *Rhesus box*, in a distance between *RHD* and *RHCE* of about 30,000 bp (Fig. 1).

Example 10: Localization of the *RHD* gene deletion in the *RHD* negative haplotypes

It was reasoned that the homology of the two *Rhesus boxes* may have been instrumental for the mechanism of the *RHD* deletion in the common *RHD* negative haplotypes. The nucleotide sequence of the *Rhesus box* in *RHD* negative DNA was determined (Fig. 5). The single *Rhesus box* detected in *RHD* negatives had a hybrid structure. The 5' end of this *Rhesus box* represented a upstream *Rhesus box*, the 3' end a downstream *Rhesus box*. It was determined that the 903 bp breakpoint region of the *RHD* deletion was located in the identity region of the *Rhesus boxes* (Fig. 4, arrow pointing to left).

Example 11: Specific detection of the *RHD* deletion by PCR

Two PCR based methods were developed for specific detection of the *RHD* gene deletion occurring in the prevalent *RHD* negative haplotypes (Fig. 6). Long-range PCR-SSP was performed using the expand long template PCR system with buffer 3 and primers rez4 (5' of upstream *Rhesus box*) and sr9 (*SMP1* exon 1). Annealing was at 60°C and extension 20 min at 68°C. PCR amplicons were resolved using a 1% agarose gel. PCR-RFLP was performed using the expand high fidelity PCR system and primers rez7 (non-specific, 5' of *Rhesus box* identity region) and rnb31 (specific for downstream *Rhesus box*, 3' of *Rhesus box* identity region). Annealing was at 65°C and extension 10 min at 68°C. PCR amplicons were digested with *PstI* for 3 hrs at 37°C and fragments resolved using a 1% agarose gel..

These techniques allowed the ready and direct detection of the common *RHD* negative haplotypes, even if they are *in trans* to *RHD* positive haplotypes. PCR-RFLP was further applied to a larger number of samples (Table 3). As expected, all 33 samples with known genotype were correctly typed. In 68 additional samples representative of the most common phenotypes, the results were consistent with the known haplotype frequencies in the population.

Example 12: *RHD* PCR-SSP

The PCR-SSP reactions (Table 4) were adapted and extended from a previously described *RHD* exon specific PCR-SSP method (Gassner, Transfusion 37:1020-6, 1997) and were triggered to work under identical thermocycling conditions. Concentrations of specific primers were 0.2 µM for all reactions with the exception of exon 6 (0.1 µM), intron 7 (0.4 µM) and exon 9 (0.4 µM). For most samples intron 4/exon 7 was tested as multiplex reaction containing 0.2 µM of exon 7 (primer set ga71/ga72) and 0.1 µM of intron 4 primers. Each reaction contained a set of HGH primers (Gassner, Transfusion 37:1020-6, 1997) as an internal control in concentrations of 0.05 µM for promoter, intron 4, and exon 7 with ga71/ga72; 0.075µM for exon 10; 0.1 µM for intron 7; 0.15 µM for exon 3, exon 4, exon 7 with rb26/re71, and exon 9; 0.2 µM for exon 5 and exon 6. Mg^{2+} concentration was 0.4 µM for intron 7 and for all other reactions 0.15 µM. For exon 6, 20 % solution Q (Qiagen, Hilden, Germany) was added.

Example 13. Improved *RHD* PCR-SSP for routine DNA typing.

Based on the alleles detected in this study and described previously, we devised an improved RHD PCR-SSP for routine DNA typing that included the specific detection of RHDΨ and alleles detected in this study, like RHD(W16X) in a single PCR tube. Reaction A contained primers ga71 and ga72 at 0.3 µM, rb12 and re41 at 0.1 µM, and HGH primers at 0.1 µM. Mg2+ was at 0.175 µM. Reaction B contained primers RhPsiF and RhPsiB at 0.5 µM, re11d and RhX1f1 at 0.3 µM, re721 and rb9 at 0.2 µM and as control primers rend9b1 and rend 9b2 at 0.2 µM. Primer sequences were ga71, gttgtaaccgagtgctggggattc; ga72, tgccggctccgacggtatc; rb12, tcctgaacctgctctgtgaagtgc; re41, cgatacccagtttgtctgccatgc; RhPsiF, agacagactaccacatgaacttac; RhPsiB, tctgatctttatcctccgttccctc; re11d, agaagatgggggaatctttttcct; RhX1f1, cgctgcctgcccctctga; re721, ctggaggctctgagaggttgag; rb9, aagctgagttccccaatgctgagg; rend9b1, cactgcacttggcaccattgag; rend9b2, ttccgaaggctgcttttccc.

The PCR reactions could be performed in two tubes (Fig. 15), tested five polymorphisms and were expected to have a false-positive rate of less than 1:10,000 (Table 11).

Example 14: PCR reactions for Cde^s

A hybrid exon 3 with a N152T substitution occurring in the Cde^s haplotype was detected by a PCR-SSP reaction using specific primers Rh152Tb and ga31 at 0.3 µM. The L245V substitution observed in Cde^s was detected with specific primers Rh223Vf and Rh245Vb at 0.2 µM. HGH primer concentrations were 0.1 µM. The other PCR conditions were identical as described in the previous paragraph. Primers sequences were Rh152Tb, gatattactgatgaccatcctcatgg; Rh223Vf, ttgtggatgttctggccaagtg; and Rh245Vb, gctgtcaccactctgactgctac. The *Cde^s* haplotype, that is frequent in Africans (Faas, Transfusion 37:38-44, 1997; Singleton, Blood 95:12-8, 2000), possesses a hybrid exon 3 harboring the *RHCE* specific N152T substitution (Faas, Transfusion 37:38-44, 1997). This hybrid exon is expected to be typed as *RHD* positive by the *RHD* exon 3 specific PCR that detected an A at position 383 (codon 128) and was used in the population survey. Since pattern 4 and pattern 8 were compatible with the known data about the *Cde^s* haplotype, the presence of a hybrid exon 3 was evaluated in the two samples by sequencing the 3' part of exon 3 and by a PCR-SSP specific for an exon 3 hybrid indicative of *Cde^s*. The pattern 4 sample possessed a normal *RHD* exon 3, while the pattern 8 sample had a hybrid exon 3 as predicted for a *Cde^s* haplotype. Also, the T at position 410 (A137V substitution) typical for the *Cde^s* haplotype (Daniels, Transfusion 38:951-8, 1998) and also present in *D category III type IV* was detected. The identity of pattern 8 and *Cde^s* was further corroborated by a PCR-SSP detecting G at position 733 (L245V substitution).

Example 15: 5' breakpoint region of *Cde^s* in intron 3.

Based on its cDNA, *Cde^s* had been characterized as an *RHD-CE(3-7)-D* hybrid gene, in which the 5' part of exon 3 derived from *RHD* and the 3' part of exon 3 including codon 152 derived from *RHCE*. We noted that a similar hybrid exon 3

with a N152T substitution was found in D category III type IV (Wagner, F.F., Frohmajer, A., Ladewig, B., Eicher, N.I., Lonicer, C.B., Müller, T.H., Siegel, M.H., and Flegel, W.A. Weak D alleles express distinct phenotypes. *Blood* 95:2699-2708, 2000) and in D category IVa (Rouillac, C., Colin, Y., Hughes-Jones, N.C., Beolet, M., D'Ambrosio, A.-M., Cartron, J.P., and Le Van Kim, C. Transcript analysis of D category phenotypes predicts hybrid Rh D-CE-D proteins associated with alteration of D epitopes. *Blood* 85:2937-2944, 1995), two aberrant *RHD* alleles in which exons 4 to 7 derived from *RHD*. We reasoned that the N152T substitution might have antedated the substitution of *RHD* exons 4 o 7 in Cde^s. In this case, the 5' breakpoint region was expected to be located in intron 3 rather than exon 3 as predicted from the cDNA. We hence evaluated the presence of *RHD* specific polymorphisms in Cde^s intron 3.

To evaluate the presence of the *Eco*RV-site at nucleotide position 752 (*RHD* specific) and 2872 (*RHCE* specific) and of the *Pvu*II-site at nucleotide position 1777 (*RHCE* specific), the 5' part of intron 3 of *RHD* and *RHCE* was amplified using primers rb3 and rb33 and digested with *Eco*RV or *Pvu*II. To evaluate the presence of the *Sac*I-site at nucleotide position 7797 (*RHCE* specific) and of the *Alw44*I-site at nucleotide position 8550 (*RHD* specific), the 3' part of intron 3 of *RHD* and *RHCE* was amplified using primers rb34 and rb5 and digested with *Sac*I or *Alw44*I. Primer sequences were rb3, aaggtcaacttggcgcagttggtgg; rb33, gtgagactgagttctgtattctgg; rb34, ccagaatacagaactcagtctcac; rb5, ggcagacaaactgggtatcgttgc.

The PCR-RFLP analysis of these intron 3 polymorphisms indicated that *RHD* specific sequences were present at least up to intron 3 position 2872. To further determine the 5' breakpoint region of Cde^s, we sequenced a DNA stretch encompassing the breakpoint region. DNA was amplified using primers rb3 and re37 and sequenced using primers rb33, rb34 and Cdesf1. Primer sequences were re37, gggttaaagtcacatacacagatg; Cdesf1, atacagaactcagtctcacaacttag. We determined that the breakpoint region was located in intron 3 as shown in Figure 12.

Example 16: 3' breakpoint region of *Cde*^{s} in intron 7.

To determine the 3' breakpoint region of *Cde*^{s} in intron 7, we sequenced parts of intron 7. DNA was amplified using sense primers rb8, re77 and rex1 and antisense primers rb51 and re711b. Primers rb43, rex19c, cdes7b2, and cdes7f2 were used for nucleotide sequencing. Primer sequences were rb8, gtgttgtaaccgagtgctgggg; re77, tctccacagctccatcatggg; rex1, ggctgtaaaaatggctgaagcag; rb51, gcatgacgtgttctgcctcttg; re711b, ctatcagcattctgatctcaacg; rb43, gaatagcagagaaaacctcagactgcc; rex19c, gctccattcttgacaatacaggc; cdes7b2, gcttatactatataagttgggtttttgg; cdes7f2, gtttgaatcccaagagccactcat. We established the breakpoint region as shown in Fig. 13. The structure of the 3' breakpoint region was intriguing, because there were multiple switches between *RHCE* and *RHD* specific sequences. Those features are unusual for a breakpoint region and may be used for specific diagnosis of *Cde*^{s}. They may indicate that the parental alleles differed from the standard *RHCE* and *RHD* sequences or that after the major gene conversion, additional small gene conversions were introduced.

Example 17: A PCR-SSP to specifically detect *Cde*^{s}.

Usually, the presence of *Cde*^{s} is identified by the *RHD-CE-D* hybrid pattern in an *RHD* exon specific PCR. Such an approach does not allow the specific detection of the D negative *Cde*^{s} haplotype, if an *RHD* positive haplotype occurs *in trans*. Since *Cde*^{s} does not contain a hybrid *Rhesus box*, a *RHD*/*Cde*^{s} heterozygous person is likely mistyped as *RHD*$^+$/*RHD*$^+$ homozygous. There are several distinct features of *Cde*^{s} in the promoter, intron 2, exon 2, and exon 3 that might be used for a specific detection. These features are, however, shared by the D positive alleles D category IVa and partially by D category III type IV, which would hence confound such methods of detection.

Based on the *Cde*^{s} specific DNA sequence in intron 7, we developed a PCR-SSP that specifically detected *Cde*^{s}. The 3' breakpoint region of *Cde*^{s} in intron 7 was detected by PCR-SSP using specific primers Cdes7f2 and Cdes7b2 at 0.4 µM and HGH control primers at 0.15 µM. The other PCR conditions were identical as described in example 12. Primer sequences were Cdes7f2,

gtttgaatcccaagagccactcat; Cdes7b2, gcttatactatataagttgggtttttgg. We obtained a specific product with the index *Cde*^{s} sample (Figure 14), two additional *Cde*^{s} samples and a *RHDΨ/Cde*^{s} heterozygous sample (Figure 14). Normal *RHD* positive and *RHD* negative samples as well as samples of D category III type IV and of D category IVa did not result in a specific PCR product (Figure 14). We concluded that our PCR-SSP method allowed a specific detection of *Cde*^{s}, even if it occurred *in trans* to another *RHD* positive allele. Furthermore, the detection method was not confounded by D category III type IV or D category IVa that shared the N152T substitution with *Cde*^{s}. It should be noted that the latter haplotypes are frequent in populations comprising African ethnic background, in which *Cde*^{s} is prevalent. The method described by us in this example allowed the specific detection of *Cde*^{s}, is not confounded by the other haplotypes and hence represents a considerable improvement to the prior art. Our characterization of the 5' breakpoint region (example 15) will likewise allow the specific detection of *Cde*^{s} by any suitable method known in the art, like PCR-SSP, PCR-LP, PCR-RFLP, PCR-SSO, Southern blotting etc.

The specific detection of *Cde*^{s} is also important for the correct prediction of the antigen C. The *RHD* gene of *Cde*^{s} encodes for an antigen C that is often missed in DNA based methods for the prediction of antigen C.

Example 18: Immunohematology

One sample of each *RHD* positive allele was evaluated by direct agglutination with two monoclonal anti-D (Seraclon anti-D, clone BS226; Biotest, Dreieich, Germany, and Frekaklon anti-D, clone MS201; Gull, Bad Homburg, Germany). Indirect antiglobulin test was done in a gel matrix test (LISS-Coombs 37 °C, DiaMed-ID Micro Typing System, DiaMed, Cressier sur Morat, Switzerland) using an oligoclonal anti-D (Seraclon anti-D blend, clones H41 11B7, BS221 and BS232; Biotest). Samples reactive in gel matrix technique were further investigated using the monoclonal anti-D HM10, HM16, P3x61, P3x35, P3x212 11F1, P3x212 23B10, P3x241, P3x249, P3x290 (Diagast, Loos, France) and H41 11B7 (Biotest). The presence of a D_{el} phenotype was determined by adsorption of 500 µl of a

polyclonal anti-D (Human incomplete anti-D; Lorne Laboratories, Reading, UK) to 500 µl red cells for 1 h at 37 °C and elution using a chloroform technique (Flegel, Transfusion 40:428-434, 2000). The analysis of samples routinely grouped as D negative revealed 16 D_{el} samples and 3 D positive samples with weak or partial D. These samples clustered among samples previously believed to be D negative with a C or E (Table 9). Nineteen of twenty-seven discrepancies between routine serology and a PCR testing intron 4 and exon 7 represented D positive samples missed by serology, only eight were due to false-positive PCR.

Example 19: Haplotype frequencies

For alleles observed more than once, their haplotype association was trivial. Alleles that were observed only once were assumed to be associated with the Cde or cdE haplotype rather than the cde haplotype, because no *RHD* positive allele was detected in any ccddee sample. An allele occurring in a single CcddEe sample was formally counted half for Cde and half for cdE. CCddee samples were assumed to harbour one aberrant and one normal Cde allele. The frequency of a given aberrant *RHD* allele in its haplotype was calculated as the number of observed samples divided by the number of the corresponding haplotypes under observation (500 Cde, 302 cdE). The population frequency of an *RHD* allele was calculated from the frequency of this allele in its haplotype and the known frequency of the haplotype in the local population (Wagner, Infusionsther. Transfusionsmed. 22:285-90, 1995). The haplotype frequencies were calculated for each PCR pattern and for each *RHD* allele (Table 8). In accordance with a previous study in England by Avent et al. (Avent, Blood 89:2568-77,1997), 4.9% of Cde haplotypes and 1.5% of cdE haplotypes were *RHD* positive in our population. As no *RHD* positive allele was detected among 314 ccddee samples, the frequency in the cde haplotype was less than 0.5 % (upper limit of one-sided 95% confidence interval, Poisson distribution). The three frequencies differed statistically significantly from each other ($p<0.05$; two sided Fisher's exact test for each pairwise comparison corrected according to Bonferoni-Holm). The population frequency of any D negative *RHD* positive haplotype was estimated to be 1:1,606. D_{el} alleles could only be observed in the presumed Cde haplotypes. About 3% of samples carrying

antigen C that were typed D-negative in the blood bank routine represented D_{el}. The population frequency of D_{el} alleles was 1:3,030.

Table 1. Primers

Primer	Nucleotide sequence	Localization	Position
rb10b	ggctaaatattttgatgaccaagtt	*RHD* cDNA	1,194 to 1,217
re011d	gcagccaacttcccctgtg	*RHD* promoter	-883 to -901
re014	gctctaccttggtcacctcc	dJ469D22	52,189 to 52,209
re04	aggtcacatccatttatcccactg	dJ469D22	53, 968 to 53,945
re11d	agaagatgggggaatctttttcct	dJ469D22	51,193 to 51,216
re96	ttgtgactgggctagaaagaaggtg	dJ469D22	242 to 216
rea7	tgttgcctgcatttgtacgtgag	*RHD* cDNA	1,311 to 1,333
rend31	ttctgtctgggttggggaggg	dJ465N24	128,649 to 128,629
rend32	ggaggggttaatatgggtggc	dJ465N24	127,355 to 127,375
rend8b1	tttgtcctggttgcctgtggtc	dJ465N24	69,296 to 69,274
rend8b2	caaatcctgttgactggtctcgg	dJ465N24	68,451 to 68,473
rend9a1	aacggctccatcacccctaaag	dJ465N24	50,008 to 49,987
rend9a2	cccactcctagataccaacccaag	dJ465N24	49,059 to 49,083
rey14a	ctttatgcactgcctcgttgaatc	dJ469D22	56,792 to 56,769
rey14b	ttgactggtgtggttgctgttg	dJ469D22	55,863 to 55,884
rey15a	gcagaaaggggagttgatgctg	dJ469D22	55,416 to 55,395
rey7	ctgacaaagttgagagcccactg	dJ469D22	62,324 to 62,346
rey8	ttaagcctacatccacatgctgag	dJ469D22	62,854 to 62,831
rez2	ccttggtctgccagaattttca	*RHD* cDNA	2738 to 2717
rez4	gtttggcatcataggagatttggc	dJ465N24	120,101 to 120,124
rez7	cctgtccccatgattcagttacc	dJ465N24	124,831 to 124,854
rh7	acgtacaaatgcaggcaac	*RHD* cDNA	1,330 to 1,312
rnb31	ccttttttgtttgtttttggcggtgc	downstream *Rhesus box*	6,710 to 6,684
rr4	agcttactggatgaccacca	*RHD* cDNA	1,541 to 1,522
sf1	gactgggggggaaaagcgcaatac	*SMP1* cDNA	142 to 164
sf1c	gtattgcgcttttccccccagtc	*SMP1* cDNA	164 to 142
sf3	tgacttgctctcatcccacatg	*SMP1* cDNA	1,696 to 1,717
sm19	gggcttgaagcaagtaaatggaag	*SMP1* intron 1	- 58 to -35
sr1	gctatcaatattttcttggttacagacac	*SMP1* cDNA	2,172 to 2,144
sr3	gttcactgccataagtcttcagtgc	*SMP1* cDNA	575 to 551
sr3kp	tggccgcactgaagacttatgg	*SMP1* cDNA	546 to 567
sr45	cagctgcatctatgataatccacc	*SMP1* cDNA	224 to243
sr47	atggacaagtccgaggtgatag	*SMP1* cDNA	315 to 344
sr47c	atcacctcggacttgtccattc	*SMP1* cDNA	342 to 321
sr5	gcaatcagagatccaaaggccaac	*SMP1* cDNA	428 to 405
sr5c	gttggcctttggatctctgattgc	*SMP1* cDNA	405 to 428
sr55	gacatagtataccctggaattgctgt	*SMP1* cDNA	472 to 497
sr55c	acagcaattccagggtatactatgtc	*SMP1* cDNA	497 to 472
sr9	ctcccccgattttagccaagaa	*SMP1* cDNA	27 to 6

For the *RHD* promoter and the *RHD* cDNA, the positions refer to the distance from the A of the start codon. For introns, they refer to the distance from the intron/exon junction. For all other sequences including the *SMP1* cDNA, they refer to the distance from the start of the published sequences. The mismatches in primers rey14b, rnb31, and sf3 were inadvertently introduced. Primers re11d, re014 and re04 do not exactly match dJ469D22, because they were designed from our raw sequences covering the 5' flanking region of *RHD*.

Table 2. Presence of *RHD* flanking sequences in the YAC 38A-A10

Primer			Amplicon size	Amplicons obtained with		
				Genomic DNA		YAC
sense	antisense	Predicted position		RHD$^+$	RHD$^-$	38A-A10
rend9a1	rend9a2 yes	*RHD* 5' flanking region	about 85,000 bp from ATG	948 bp	yes	yes
rend8b1	rend8b2 yes	*RHD* 5' flanking region	about 50,000 bp from ATG	845 bp	yes	yes
rea7	rez2 yes	*RHD* 3' flanking region	about 1,500 bp from STOP	1,412 bp	yes	no
rend32	sr9 yes	*RHCE* 3' flanking region	about 20,000 bp from STOP	1,989 bp	yes	yes
sr1	sf3 yes	*RHCE* 3' flanking region	about 1,000 bp from STOP	477 bp	yes	yes
rey14b	rey14a no	*RHCE* 5' flanking region	about 5,300 bp from ATG	929 bp	yes	yes
rey7	rey8 no	*RHCE* 5' flanking region	about 10,000 bp from ATG	530 bp	yes	yes

Table 3. PCR-RFLP for the specific detection of the *RHD* deletion

Phenotype	Known genotype	Samples tested (n)	Number of samples with *RHD* genotype: determined +/+	determined +/-	determined -/-	expected* +/+	expected* +/-	expected* -/-	P¶
Known genotype									
ccddee	cde/cde	14	0	0	14	0	0	14	N.A.
CCddee	Cde/Cde†	5	0	0	5	0	0	5	N.A.
ccddEE	cdE/cdE†	1	0	0	1	0	0	1	N.A.
D variants	D/cde‡	9	0	9	0	0	9	0	N.A.
ccDEe	cDe/cDE§	4	4	0	0	4	0	0	N.A.
Common phenotypes									
CcDee		10	1	9	0	0.5	9.5	0	>0.4
ccDEe		10	0	10	0	0.3	9.7	0	>0.5
ccDee		10	1	9	0	0.5	9.5	0	>0.4
CCDee		10	9	1	0	9.5	0.5	0	>0.4
CcDEe		12	11	1	0	11	1	0	>0.5
ccDEE		10	10	0	0	9.2	0.8	0	>0.4
CCDEe		6	5	1	0	5.8	0.2	0	>0.1

* Expected number of *RHD*$^+$/*RHD*$^+$ and *RHD*$^+$/*RHD*$^-$ samples based on known genotypes or the haplotype frequencies in the local population [41]

† *RHD*-negative in PCR.

‡ *RHD*$^+$/*RHD*$^-$, because a weak or partial D phenotype would be masked in a *RHD*$^+$/*RHD*$^+$ gentoype. These samples were weak D type 1 (n=2), type 2 (n=2), type 3 (n=2), type 4 (n=2) and D^{VII} (n=1).

§ Presence of two *RHD* genes differing in their polymorphic *Hae*III-site in intron 3 [42] demonstrated by PCR-RFLP.

N.A. - not applicable. Probabilities were calculated based on confidence limits of binomial distribution.

Table 4. *RHD* PCR-SSP

Region	Name* Reference	DNA sequence	Position		Polymorphisms detected	Amplicon size	
Promoter	re012	tccactttccacctccctgc	Promoter	-1,137 to -1,119	7 bp deletion at - 1125	255	43
	re011d	gcagccaacttcccctgtg	Promoter	-883 to -901	4 bp deletion at -896		44
Exon 3	ga31 (D-3-383)	ttgtcggtgctgatctcagtgga	Exon 3	361 to 383	383 A	154	21
	rb21	aggtccctcctccagcac	Intron 3	28 to 11			42
Exon 4	ga41 (D-4-527)	acatgatgcacatctacgtgttcgc	Exon 4	503 to 527		123	21
	ga42 (D-4-602)	cagacaaactgggtatcgttgctg	Exon 4	625 to 602	602 C		21
Intron 4	re41	cgatacccagtttgtctgccatgc	Exon 4	608 to 631		226	
	this study						
	rb12	tcctgaacctgctctgtgaagtgc	Intron 4	198 to 175	Intron 4 deletion in *RHD*		40
Exon 5	rb24	agacctttggagcaggagtg	Intron 4	-53 to -34		228	40
	ga51 (D-5-787)	ctgctcaccttgctgatcttccc	Intron 5/Exon 5		8 to 787	787 G	21
Exon 6	ga62 (D-6-826)	ttatgtgcacagtgcggtgttgg	Exon 6	804 to 826		133	21
	ga61 (D-6-916)	caggtacttggctcccccgac	Exon 6	936 to 916	916 G		21
Exon 7†	ga71 (D-7-967)	gttgtaaccgagtgctggggattc	Exon 7	944 to 967		123	21
	ga72 (D-7-1048)	tgccggctccgacggtatc	Exon 7	1,066 to 1,048	1,048 G		21
Exon 7†	rb26	aggggtgggtagggaatatg	Intron 6	-62 to -43		130	42
	re71	acccagcaagctgaagttgtagcc	Exon 7	1,008 to 985	985/986 GG		42
Intron 7	rb52	ccaggttgttaagcattgctgtacc	Intron 7	6,666 to 6,690	6,690 C		42
	rb51	gcatgacgtgttctgcctcttg	Intron 7	6,734 to 6,713	6,713 C	169	
	this study						
Exon 9	re83	gagattaaaaatcctgtgctcca	Intron 8	-56 to -34		119	42
	re94	cttggtcatcaaaatatttagcct	Exon 9	1,216 to 1,193	1,193 A		
	this study						
Exon 10 (3' UTR)	rea7	tgttgcctgcatttgtacgtgag	3'UTR	1,310 to 1,333	*RHD/Rhesus box*	*23*	44
	rr4	agcttactggatgaccacca	3'UTR	1,541 to 1,522	junction		42

* Primer names in brackets are as described by Gassner et al. [21].
† Primer set ga71/ga72 was used for the screening, primer set rb26-re71 for *RHD* exon specific PCR-SSP.

Table 5. Population survey of known D negative blood donors screened by *RHD* PCR-SSP

Documented phenotype	Samples (n)	
	screened	PCR-SSP positive
ccddee	314	0
Ccddee	433	34
ccddEe	271	5
CCddee	24	4
CcddEe	19	4
ccddEE	6	1
CcddEE	1	0
Total	1,068	48

Positive for at least one of four *RHD* specific polymorphisms tested (promoter, intron 4, exon 7 or 3' UTR).

Table 6. PCR patterns compatible with *RHD-RHCE-RHD* hybrid genes or partial *RHD* deletions in 25 D negative samples

PCR pattern	*RHD* specific PCR-SSP* P E3E4 I4 E5E6E7 I7 E9E10	Samples Possible cause†	Phenotype (n)	Documented	Confirmed	Haplotype	Reference‡
Pattern 1 Africans [24]	+ - - - - - - - - +	*RHD-CE(3-9)-D*	11	Ccddee§	D negative	Cde	Whites [1,25],
Pattern 2	+ - - - - - - - + +	*RHD-CE(3-7)-D*	4	Ccddee	D negative	Cde	this study
Pattern 3	+ + - - - - - - + +	*RHD-CE(4-7)-D*	3	ccddEe	D negative	cdE	Whites [16]
Pattern 4	+ + - - - - - + + +	*RHD-CE(4-7)-D*	1	CcddEe	D negative	n.k.¶	this study
Pattern 5	+ + - - - + + + + +	*RHD-CE(4-5)-D*	2	ccddEe$^{\alpha}$	partial D/$D_{el}$$^{\alpha}$	cDE	Whites [3, 22,30,40]
Pattern 6	+ + + + + + + - - +	*RHD-CE(8-9)-D*	3	CCddee	D negative	Cde	Whites [21]
Pattern 7	- - - - - - - - - +	*RHCE(1-9)-D(10)*	1	ccddEe	D negative	cdE	this study
Pattern 8	- + - - - - - + + +	*RHD(1-3)-CE(4-7)-D*	1	CcddEe	D negative	Cde$^{\beta}$	Africans [5,15]

* P - Promoter, E3 - Exon 3, E4 - Exon 4, I4 - Intron 4, E5 - Exon 5, E6 - Exon 6; E7 - Exon 7; I7 - Intron 7; E9 - Exon 9; E10 - Exon 10 (3' UTR)

† Assuming the presence of a single *RHD-CE-D* hybrid allele.

‡ Previously described alleles that fit PCR pattern and haplotype.

§ 11 samples: 9 Ccddee, 1 CCddee, 1 CcddEe

¶ n.k. - not known.

$^{\alpha}$ 2 samples, 1 labeled CcddEe with D_{el} phenotype, 1 labeled ccddEe with partial D D^{VI} phenotype.

$^{\beta}$ Probably identical to Cde^{s} (see below).

Table 7. *RHD* alleles with single nucleotide substitutions in 22 D negative samples

Allele	Substitution Reference	Effect(s)	Samples (n)	Phenotype		Haplotype	
				Documented	Confirmed		
RHD(W16X) study	G->A at 48	Stop codon at codon 16	2	Ccddee	D negative	Cde	this
RHD(G486(+1)A) study	g->a at 486+1	5' splice site intron 3 ACgt->ACat	3	Ccddee	D_{el}	CDe	this
RHD(G212V) study	G->T at 635	3' splice site intron 4 agGC->agTC	1	Ccddee	D negative	Cde	this
RHD(C285Y) study[1]	G->A at 854	Missense mutation G212V Missense mutation C285Y	1	ccddEe	partial D*	cDE	this
RHD(M295I)	G->T at 885	Missense mutation M295I	7	Ccddee	D_{el}	CDe†	42
RHD(Y330X) study	C->G at 985	Stop codon at codon 330	1	Ccddee	D negative	Cde	this
RHD(G1153(+1)A) study	g->a at 1153+1	5' splice site intron 8 AGgt->AGat	1	Ccddee	D negative	Cde	this
RHD(G385A)	G->C at 1154	3' splice site intron 8 agGT->agCT Missense mutation G385A	1	CcddEe	weak D	cDE	42
RHD(K409K) study	G->A at 1227	5' splice site intron 9 AGgt->AAgt	5	Ccddee	D_{el}	CDe	this

* A detailed serologic analysis of this sample representing the partial D DIM has been published previously [43].
† The same allele occurring in a cDe haplotype has been described as weak D type 11.

Table 8. Estimated frequencies in population

PCR pattern/Allele	Frequency among Cde/ cdE	Frequency in population
Pattern 1	1:45	1:4,132
Pattern 2	1:125	1:11,364
Pattern 3	1:101	1:17,976
Pattern 4	1:500*	1:45,455*
Pattern 6	1:167	1:15,152
Pattern 7	1:302	1:53,929
Pattern 8	1:500	1:45,455
RHD(W16X)	1:250	1:22,727
RHD(G212V)	1:500	1:45,455
RHD(Y330X)	1:500	1:45,455
RHD(G1153(+1)A)	1:500	1:45,455
Any D negative	1:20 / 1:67†	1:1,607
RHD(G486(+1)A)	1:167	1:15,152
RHD(M295I)	1:71	1:6,493
RHD(K409K)	1:100	1:9,091
Any D_{el}	1:33‡	1:3,030

* Assuming a Cde haplotype; a cdE haplotype would result in a frequency of 1:302 among cdE and 1:53,929 in the population. For statistics and sum frequencies, the haplotype was formally counted as 0.5 Cde and 0.5 cdE.

† 1:20 among Cde, 1:67 among cdE.

‡ 1:33 relative to the Cde haplotype.

Table 9 False negative rate in routine typing for antigen D

Documented Phenotype	Samples (n)	Confirmed phenotype (n)		False negatives	
		D_{el}	partial or weak D	n	Rate
ccddee	314	0	0	0	0%[†]
Ccddee	433	15	0	15	3.5%
ccddEe	271	0	2	2	0.7%
other[*]	50	1	1	2	4%
D negative	N.A.[‡]	0.15%	0.02%		0.17%

[*] CCddee, CcddEe, ccddEE, and CCddEe

[†] Upper limit of 95% confidence interval was 0.95% (Poisson distribution)

[‡] N.A. - not applicable. The frequencies are estimates based on the phenotype frequencies in the population [41].

Table 10 Previously described D negative, *RHD positive* alleles

Allele	Haplotype	Population	Possible match
RHD(Q41X) [2]	Cde	Whites	not detected
RHD-CE(2-9)-D [1,24,25]	Cde	Whites[1,25], Blacks[24]	Pattern 1
RHD-CE(3:455-7)-D [5,15]	Cde^s	Blacks	Pattern 8
RHD(488del4) [1]	Cde	Whites	not detected
RHD-CE(4-7)-D [16]	cdE	Whites	Pattern 3 or 4
RHDΨ [38]	cde	Blacks	not detected
RHD(600del) [10]	Cde	somatic mutation*	not detected
RHD (exon 5 variant) [8]	cde	not communicated	not detected
RHD(G314V) [34]	Cde	Japanese	not detected
RHD(exon 9 variant)	Cde	Whites	Pattern 6

* Allele acquired by somatic mutation in a woman with chronic myelogenic leucemia and restricted to the myeloid lineage

Table 11 Population rates of false positives and positive predictive value of different *RHD* PCR strategies

PCR strategy	Rate of false positives	Positive predictive value of positive result	Number of polymorphism tested
Exon 10 only	1:1,275	0.999216	1
Intron 4/Exon 7	1:4,081	0.999755	2
Intron 4/Exon 7/*RHDΨ*	1:4,700	0.999787	3
Intron 4/Exon 7/*W*16X	1:5,212	0.999808	3
Intron 4/Exon 7/Intron 7	1:6,051	0.999835	3
Exons 3, 4, 5, 6, 7, 9	1:6,051	0.999835	6
Exons 2, 3, 4, 5, 6, 7, 9, 10	1:6,051	0.999835	8
Intron 4/Exon 7/*W*16X/*RHDΨ*	1:6,267	0.999840	4
All Exons/*RHDΨ*	1:7,520	0.999867	9
Intron 4/Exon 7/Intron 7/*W*16X	1:8,921	0.999888	4
Intron 4/Exon 7/Intron 7/*W*16X/*RHDΨ*	1:12,533	0.999920	5

References

1. Andrews KT, Wolter LC, Saul A, Hyland CA: The RhD- trait in a white patient with the RhCCee phenotype attributed to a four-nucleotide deletion in the *RHD* gene. Blood 92:1839, 1998

2. Avent ND, Martin PG, Armstrong-Fisher SS, Liu W, Finning KM, Maddocks D, Urbaniak SJ: Evidence of genetic diversity underlying Rh D negative, weak D (D^u) and partial D phenotypes as determined by multiplex PCR analysis of the RHD gene. Blood 89:2568, 1997

3. Avent ND, Liu W, Jones JW, Scott ML, Voak D, Pisacka M, Watt J, Fletcher A. Molecular analysis of Rh transcripts and polypeptides from individuals expressing the D^{VI} variant phenotype: an *RHD* gene deletion event does not generate all D^{VI}ccEe phenotypes. Blood 1997;89:1779-86.

4. Blumenfeld OO, Huang CH: Molecular genetics of the glycophorin gene family, the antigens for MNSs blood groups: multiple gene rearrangements and modulation of splice site usage result in extensive diversification. Hum Mutat 6:199, 1995

5. Blunt T, Daniels G, Carritt B: Serotype switching in a partially deleted *RHD* gene. Vox Sang 67:397, 1994

6. Bowman J: The management of hemolytic disease in the fetus and newborn. Semin Perinatol 21:39, 1997

7. Carritt B, Kemp TJ, Poulter M: Evolution of the human RH (rhesus) blood group genes: a 50 year old prediction (partially) fulfilled. Hum Mol Genet 6:843, 1997

8. Carritt B, Steers FJ, Avent ND. Prenatal determination of fetal RhD type. Lancet 1994;344:205-6.

9. Cherif-Zahar B, Mattei MG, Le Van Kim C, Bailly P, Cartron JP, Colin Y: Localization of the human Rh blood group gene structure to chromosome region 1p34.3-1p36.1 by in situ hybridization. Hum Genet 86:398, 1991

10. Cherif-Zahar B, Bony V, Steffensen R, Gane P, Raynal V, Goosens D, Laursen JS, Varming K, Jersild C, Cartron JP. Shift from Rh-positive to Rh-negative phenotype caused by a somatic mutation within the RHD gene in a patient with chronic myelocytic leukaemia. Br J Haematol 1998;102:1263-70.

11. Colin Y, Cherif-Zahar B, Le Van Kim C, Raynal V, van Huffel V, Cartron J-P. Genetic basis of the RhD-positive and RhD-negative blood group polymorphism as determined by southern analysis. Blood 1991;78:2747-52.

12. Cossu G, Angius A, Gelfi C, Righetti PG: Rh D/d genotyping by quantitative polymerase chain reaction and capillary zone electrophoresis. Electrophoresis 17:1911, 1996

13. Daniels GL, Faas BH, Green CA, Smart E, Maaskant-van Wijk PA, Avent ND, Zondervan HA, von dem Borne AE, van der Schoot CE: The VS and V blood group polymorphisms in Africans: a serologic and molecular analysis. Transfusion 1998;38:951-8.

14. Döscher A, Schunter F, Müller TH: Quantitativae PCR to assess *RHD* zygosity. Infusionsther Transfusionsmed 26(suppl 1):31, 1999 (abstr.)

15. Faas BHW, Becker EAM, Wildoer P, Ligthart PC, Overbeeke MAM, Zondervan HA, von dem Borne AEGK, van der Schoot CE: Molecular background of VS and weak C expression in blacks. Transfusion 37:38, 1997

16. Faas BHW, Beckers EAM, Simsek S, Overbeeke MAM, Pepper R, van Rhenen DJ, von dem Borne AEGK, van der Schoot CE: Involvement of Ser103 of the Rh polypeptides in G epitope formation. Transfusion 36:506, 1996

17. Filbey D, Hanson U, Wesstrom G: The prevalence of red cell antibodies in pregnancy correlated to the outcome of the newborn: a 12 year study in central Sweden. Acta Obstet Gynecol Scand 74:687, 1995

18. Flegel WA, Khull SR, Wagner FF. Primary anti-D immunization by weak D type 2 RBCs. Transfusion 2000; 40: 428 - 434

19. Flegel WA, Wagner FF, Müller TH, Gassner C: Rh phenotype prediction by DNA typing and its application to practice. Transfus Med 8:281, 1998

20. Fujiwara H, Okuda H, Omi T, Iwamoto S, Tanaka Y, Takahashi J, Tani Y, Minakami H, Araki S, Sato I, Kajii E: The STR polymorphisms in intron 8 may provide information about the molecular evolution of *RH* haplotypes. Hum Genet 104:301, 1999

21. Gassner C, Schmarda A, Kilga-Nogler S, Jenny-Feldkircher B, Rainer E, Müller TH, Wagner FF, Flegel WA, Schönitzer D: RhesusD/CE typing by polymerase chain reaction using sequence-specific primers. Transfusion 37:1020, 1997

22. Huang CH. Human D^{VI} category erythrocytes: correlation of the phenotype with a novel hybrid RhD-CE-D gene but not an internally deleted RhD gene. Blood 1997;89:1834-9.

23. Huang CH, Reid ME, Chen Y, Coghlan G, Okubo Y: Molecular definition of red cell Rh haplotypes by tightly linked *Sph*I RFLPs. Am J Hum Genet 58:133, 1996

24. Huang CH: Alteration of RH gene structure and expression in human dCCee and DCW- red blood cells: phenotypic homozygosity versus genotypic heterozygosity. Blood 88:2326, 1996

25. Hyland CA, Wolter LC, Saul A. Three unrelated Rh D gene polymorphisms identified among blood donors with Rhesus CCee (r'r') phenotypes. Blood 1994;84:321-4.

26. Kemp TJ, Poulter M, Carritt B: Microsatellite variation within the human *RHCE* gene. Vox Sang 77:159, 1999

27. Lo Y-MD, Bowell PJ, Selinger M, Mackenzie IZ, Chamberlain P, Gillmer MDG, Littlewood TJ, Fleming KA, Wainscoat JS: Prenatal determination of fetal RhD status by analysis of peripheral blood of rhesus negative mothers. Lancet 341:1147, 1993

28. Maaskant-van Wijk PA, Hemker MB, Douglas-Berger L, et al. Ethnic variability of the Rhesus system. Transfusion 1999;39:(10S)103S-4S (Abstract).

29. Maaskant-van Wijk PA, Faas BHW, de Ruijter JAM, Overbeeke MAM, von dem Borne AEGK, van Rhenen DJ, van der Schoot CE. Genotyping of RHD by multiplex

polymerase chain reaction analysis of six *RHD*-specific exons. Transfusion 1998;38:1015-21.

30. Maaskant-van Wijk PA, Beckers EAM, van Rhenen DJ, Mouro I, Colin Y, Cartron J-P, Faas BHW, van der Schoot CE, Apoil PA, Blancher A, et al. Evidence that the RHD(VI) deletion genotype does not exist. Blood 1997;90:1709-11.

31. MacGeoch C, Mitchell CJ, Carritt B, Avent ND, Ridgwell K, Tanner MJ, Spurr NK: Assignment of the chromosomal locus of the human 30-kDal Rh (rhesus) blood group-antigen-related protein (Rh30A) to chromosome region 1p36.13----p34. Cytogenet Cell Genet 59:261, 1992

32. Mourant AE, Kopec AC, Domaniewska-Sobczak K: The distribution of the human blood groups and other polymorphisms. London, Oxford University Press, 1976

33. Okuda H, Suganuma H, Tsudo N, Omi T, Iwamoto S, Kajii E: Sequence analysis of the spacer region between the *RHD* and *RHCE* genes. Biochem Biophys Res Commun 263:378, 1999

34. Okuda H, Kawano M, Iwamoto S, Tanaka M, Seno T, Okubo Y, Kajii E: The RHD gene is highly detectable in RhD-negative Japanese donors. J Clin Invest 100:373, 1997

35. Onda M, Fukuda M: Detailed physical mapping of the genes encoding glycophorins A, B and E, as revealed by P1 plasmids containing human genomic DNA. Gene 159:225, 1995

36. Reboul J, Gardiner K, Monneron D, Uze G, Lutfalla G: Comparative genomic analysis of the interferon/interleukin-10 receptor gene cluster. Genome Res 9:242, 1999

37. Salvignol I, Calvas P, Socha WW, Colin Y, Le Van Kim C, Bailly P, Ruffie J, Cartron JP, Blancher A: Structural analysis of the *RH*-like blood group gene products in nonhuman primates. Immunogenetics 41:271, 1995

38. Singleton BK, Green CA, Avent ND, Martin PG, Smart E, Daka A, Narter-Olaga EG, Hawthorne LM, Daniels G: The presence of an *RHD* pseudogene containing a 37 base pair duplication and a nonsense mutation in africans with the Rh D- negative blood group phenotype. Blood 95:12, 2000

39. Urbaniak SJ, Robertson AE: A successful program of immunizing Rh-negative male volunteers for anti-D production using frozen/thawed blood. Transfusion 21:64, 1981

40. Wagner FF, Gassner C, Müller TH, Schönitzer D, Schunter F, Flegel WA: Three molecular structures cause Rhesus D category VI phenotypes with distinct immunohematologic features. Blood 91:2157, 1998

41. Wagner FF, Kasulke D, Kerowgan M, Flegel WA: Frequencies of the blood groups ABO, Rhesus, D category VI, Kell, and of clinically relevant high-frequency antigens in South-Western Germany. Infusionsther Transfusionsmed 22:285, 1995

42. Wagner FF, Gassner C, Müller TH, Schönitzer D, Schunter F, Flegel WA: Molecular basis of weak D phenotypes. Blood 93:385, 1999

43. Wagner FF, Frohmajer A, Ladewig B, Eicher NI, Lonicer CB, Müller TH, Siegel MH, Flegel WA. Weak D alleles express distinct phenotypes. Blood 2000; 95: 2699 – 2708

44. Wagner FF, Flegel WA. *RHD* gene deletion occurred in the *Rhesus box*. Blood 2000;

45. Wissenschaftlicher Beirat der Bundesärztekammer; Paul-Ehrlich-Institut. Richtlinien zur Blutgruppenbestimmung und Bluttransfusion (Hämotherapie). Köln: Deutscher Ärzte-Verlag; 1996.

46. Legler TJ, Kohler M, Mayr WR, Panzer S, Ohto H, Fischer GF; Genotyping of the human platelet antigen systems 1 through 5 by multiplex polymerase chain reaction and ligation-based typing. Transfusion 1996 May; 36(5): 426-31

Claims

1. A nucleic acid molecular structure representing the *Rhesus* genes locus comprising the *RHD*, *SMP1*, and *RHCE* genes and/or the *Rhesus box(es)*, preferably the hybrid *Rhesus box*, the upstream *Rhesus box* and/or the downstream *Rhesus box*, the sequence of which is shown in Figures 8 to 10.

2. The nucleic acid molecular structure of claim 1 representative of the common *RHD* negative haplotypes.

3. A nucleic acid molecular structure, dubbed *Rhesus box*, which is flanking the breakpoint region of the *RHD* deletion in the common *RHD* negative haplotypes.

4. The nucleic acid molecular structure of claim 1 representative of an *RHD* negative haplotype comprising an *RHD* gene deletion involving the upstream *Rhesus box*, the downstream *Rhesus box* or both.

5. A nucleic acid molecular structure flanking the *Rhesus box* in the common *RHD* negative haplotypes.

6. The nucleic acid molecular structure of claim 1 representative of the common *RHD* positive haplotypes.

7. The nucleic acid molecular structure of claim 1 derived from a sample comprising an *RHD* positive haplotype that is serologically classified RhD negative.

8. The nucleic acid molecular structure of claim 7 wherein said sample is selected from a Caucasian population.

9. The nucleic acid molecular structure of claim 7 or 8 comprising partial *RHD*-deletions or substitutions.

10. The nucleic acid molecular structure of claim 9 comprising deletions or substitutions of *RHD* exons 3 to 7, or 4 to 7 giving rise to a CcddEe phenotype, or 1 to 9.

11. The nucleic acid molecular structure of claim 9 comprising an *RHD-CE-D* hybrid allele, which is representative of a *Cde*^{s} haplotype but also occurs in other Rhesus haplotypes, carrying a 5' breakpoint region located in intron 3, the sequence of which breakpoint region is shown in Figure 12, and/or a 5' breakpoint region located in intron 7, the sequence of which breakpoint region is shown in Figure 13, or both breakpoint regions.

12. The nucleic acid molecular structure of claims 1, 6, 7 or 8 comprising an *RHD-CE(3-7)-D* hybrid allele, an *RHD-CE(4-7)-D* hybrid allele giving rise to a CcddEe phenotype or an *RHCE(1-9)-D(10)* hybrid allele.

13. The nucleic acid molecular structure of claim 11 wherein the *RHD-CE-D* hybrid allele of claim 11 encodes a polypeptide having antigen C reactivity.

14. The nucleic acid molecular structure of any one of claims 6 to 8 or a nucleic acid molecule being derived from the *RHD* gene comprising a single nucleotide substitution within the coding region of the *RHD* gene or within a 5' or 3' splice site.

15. The nucleic acid molecular structure or a nucleic acid molecule of claim 14 wherein said nucleotide substitution gives rise to a stop-codon at codon 16.

16. The nucleic acid molecular structure or a nucleic acid molecule of claim 15 wherein said substitution gene gives rise to an *RHD(W*16X*)* mutation.

17. The nucleic acid molecular structure or a nucleic acid molecule of claim 16 wherein said substitution is a G→A substitution at nucleotide position 48.

18. The nucleic acid molecular structure or a nucleic acid molecule of claim 14 wherein said nucleotide substitution gives rise to a stop codon at codon 330.

19. The nucleic acid molecular structure or a nucleic acid molecule of claim 18 wherein said substitution gives rise to a *RHD*(Y330X) mutation.

20. The nucleic acid molecular structure or a nucleic acid molecule of claim 19 wherein said substitution is a C → G substitution at nucleotide position 985.

21. The nucleic acid molecular structure or a nucleic acid molecule of claim 14 wherein said substitution gives rise to a missense mutation at codon 212.

22. The nucleic acid molecular structure or a nucleic acid molecule of claim 21 wherein said substitution gives rise to a *RHD(*G212V*)* missense mutation.

23. The nucleic acid molecular structure or a nucleic acid molecule of claim 22 wherein said substitution is a G→T substitution at position 635.

24. The nucleic acid molecular structure or a nucleic acid molecule of claim 14 wherein said substitution gives rise to a mutation within a 4-nucleotide sequence, a 6-nucleotide sequence or an 8-nucleotide sequence comprising the consensus splice site at the exon8/intron8 boundary.

25. The nucleic acid molecular structure or a nucleic acid molecule of claim 24 wherein said substitution give rise to a *RHD(G1153(+1)A)* mutation.

26. The nucleic acid molecular structure or a nucleic acid molecule of claim 25 wherein said substitution is a substitution at the 5' splice site intron 8 from AGgt to AGat.

27. The nucleic acid molecular structure or a nucleic acid molecule of any one of claims 15 to 26 correlating with a RhD negative phenotype.

28. The nucleic acid molecular structure or a nucleic acid molecule of claim 14 wherein said substitution gives rise to a mutation within a 4-nucleotide sequence, a 6-nucleotide sequence or an 8-nucleotide sequence comprising the consensus splice site at the exon3/intron3 boundary.

29. The nucleic acid molecular structure or a nucleic acid molecule of claim 28 wherein said substitution gives rise to a *RHD(G486(+1)A)* mutation.

30. The nucleic acid molecular structure or a nucleic acid molecule of claim 29 wherein said substitution is a substitution at the 5' splice site intron 3 from ACgt to ACat.

31. The nucleic acid molecular structure or a nucleic acid molecule of claim 14 wherein said substitution gives rise to a mutation within a 4-nucleotide sequence, a 6-nucleotide sequence or an 8-nucleotide sequence comprising the consensus splice site at the exon9/intron9 boundary.

32. The nucleic acid molecular structure or a nucleic acid molecule of claim 31 wherein said substitution gives rise to a *RHD(*K409K*)* mutation.

33. The nucleic acid molecular structure or a nucleic acid molecule of claim 32 wherein said substitution is a substitution at the 5' splice site intron 9 from AGgt to AAgt.

34. The nucleic acid molecular structure or a nucleic acid molecule of claims 28 to 33 correlating with a D_{el}-phenotype.

35. A process to specifically detect a *RHD* negative haplotype in a sample by utilizing the *RHD*, *SMP1* genes and/or the *Rhesus box(es)*, preferably the *hybrid Rhesus box*, the upstream *Rhesus box* and/or the downstream *Rhesus box*, the sequence of which is shown in Figures 8 to 10 or any structural feature or nucleotide sequence or both of any one of claims 2 to 34 or combinations thereof with techniques known in the art, preferably by PCR-RFLP, PCR-SSP or long-range PCR.

36. A process to specifically detect a common *RHD* negative haplotype comprising the following steps:

(a) isolating the DNA from a blood sample or blood donor

(b) hybridizing at least two oppositely oriented primers under stringent conditions to the DNA so as to carry out a PCR

(c) amplifying the target sequence

(d) separating the amplification products on a gel

(e) analyzing the amplicons

37. A process to specifically detect a common *RHD* negative haplotype in a sample comprising the detection of the hybrid *Rhesus box*.

38. A process to specifically detect a common *RHD* negative haplotype in a sample comprising assessing the molecular nucleic acid structure comprising the hybrid *Rhesus box* and the flanking regions thereof.

39. A process to specifically detect a *RHD* negative haplotype in a sample comprising the step of detecting any of the breakpoint regions mentioned in claim 11.

40. The process of claim 37 or 38 wherein said detection or assessment comprises the determination of the length of a nucleic acid molecule comprising the hybrid *Rhesus box* or parts thereof.

41. The process of any one of claims 37 to 40 wherein said detection or assessment is effected by PCR-RFLP, PCR-SSP or long-range PCR or by a probe specifically hybridizing to the hybrid *Rhesus box*, to the breakpoint or breakpoint region depicted in Figure 4 or 5 or 12 or 13 or hybridizing to the upstream or downstream *Rhesus box* in a Southern blot analysis.

42. The process of claim 41 wherein said probe hybridizes to the breakpoint or breakpoint region depicted in Figure 4 or 5 or 12 or 13.

43. The process of claim 41 or 42 wherein detection of said hybridization is effected by Southern blot analysis, gel-electrophoresis, biochip-analysis, fluorescence or molecular weight determination.

44. A vector comprising the nucleic acid molecular structure or a nucleic acid molecule of any one of claims 1 to 34.

45. A non-human host transformed with the vector of claim 44.

46. A method of producing a protein product of the *RHD* gene comprising culturing the host of claim 45 under suitable conditions and isolating the Rhesus protein produced.

47. A protein product of the *RHD* gene encoded by the nucleic acid molecule or structure of any one of claims 1 to 34 or produced by the method of claim 46.

48. An oligonucleotide hybridizing under stringent conditions to a portion of the nucleic acid molecular structure or the nucleic acid molecule of any one of claims 1 to 34 wherein said portion comprises said (missense) mutation or

said stop codon or to the complementary portion thereof or hybridizing to a region involving the breakpoint of said hybrid gene.

49. An antibody or aptamer or phage specifically binding to the protein product of the *RHD* gene of claim 47.

50. A method to simultaneously detect the presence of *RHDΨ* and any of the *RHD* molecular structures of any one of claims 1 to 34 comprising hybridizing the oligonucleotide of claim 48 and at least an other oligonucleotide hybridizing to a *RHDΨ* structure under stringent conditions to nucleic acid molecules comprised in the sample obtained from a human and detecting said hybridization.

51. A method for testing for the presence of a nucleic acid molecule encoding a mutant Rhesus D antigen or of a nucleic acid molecule carrying a deletion of the *RHD* gene as characterized by the nucleic acid molecular structure or a nucleic acid molecule of any one of claims 1 to 34 in a sample comprising hybridizing the oligonucleotide of claim 48 under stringent conditions to nucleic acid molecules comprised in the sample obtained from a human and detecting said hybridization.

52. The method of claim 51 further comprising digesting the product of said hybridization with a restriction endonuclease and analyzing the product of said digestion.

53. A method for testing simultaneously for the presence of *RHDΨ* and any of the *RHD* molecular structures of any one of claims 1 to 34 in a sample comprising determining the nucleic acid sequence of at least a portion of the nucleic acid molecular structure or nucleic acid molecule of any one of claims 1 to 34, said portion encoding said (missense) mutation, said stop codon or a breakpoint of said hybrid gene and determining of at least a portion of a *RHDΨ* structure.

54. A method for testing for the presence of a nucleic acid molecule encoding a mutant Rhesus D antigen or of a nucleic acid molecule carrying a deletion of the *RHD* gene as characterized by the nucleic acid molecular structure or a nucleic acid molecule of any one of claims 1 to 34 in a sample comprising determining the nucleic acid sequence of at least a portion of the nucleic acid molecular structure or nucleic acid molecule of any one of claims 1 to 34, said portion encoding said (missense) mutation, said stop codon or a breakpoint of said hybrid gene.

55. The method of claim 54 further comprising, prior to determining said nucleic acid sequence, amplification of at least said portion of said nucleic acid molecule or structure.

56. A method for testing simultaneously for the presence of *RHDΨ* and any of the *RHD* molecular structures of any one of claims 1 to 34 in a sample comprising carrying out an amplification reaction using a set of primers that amplifies at least a portion of said sequence wherein at least one of the primers employed in said amplification reaction is the oligonucleotide of claim 48 and at least a primer amplifying a *RHDΨ* structure and analysing the amplified product(s).

57. A method for testing for the presence of a nucleic acid molecule encoding a mutant Rhesus D antigen or of a nucleic acid molecule carrying a deletion of the *RHD* gene as characterized by the nucleic acid molecular structure or nucleic acid molecule of any one of claims 1 to 34 in a sample comprising carrying out an amplification reaction using a set of primers that amplifies at least a portion of said sequence wherein at least one of the primers employed in said amplification reaction is the oligonucleotide of claim 48.

58. The method of claim 57 wherein at least one of the primers employed in said amplification reaction is the oligonucleotide of claim 48.

59. The method of any one of claims 55 to 58 wherein said amplification is effected by or said amplification reaction is the polymerase chain reaction (PCR).

60. The method of claim 59 wherein said PCR is PCR-RFLP, PCR-SSP or long-range PCR.

61. The method of any one of claims 55 to 60 wherein the molecular weight of the amplification product is analyzed.

62. A method for testing for the presence of the nucleic acid molecular structure or nucleic acid molecule of any one of claims 7 to 34 encoding *RHD* positive alleles comprising the following steps:

(a) isolating the DNA from a blood sample or blood donor;

(b) hybridizing at least two oppositely oriented primers under stringent conditions to the DNA so as to carry out a PCR;

(c) amplifying the target sequence;

(d) separating the amplification products on a gel; and

(e) analyzing the amplicons.

63. The method of claim 62 wherein said *RHD* positive alleles are derived from a serologically RhD negative sample.

64. The method of claim 62 or 63 wherein said sample is selected from a Caucasian population.

65. A method for testing for the presence of a protein product of the *RHD* gene of claim 47 in a sample comprising assaying a sample obtained from a human for specific binding to the antibody or aptamer or phage of claim 49.

66. A method for testing for the presence of a protein product of the *RHD* gene encoding the nucleic acid molecular structure or nucleic acid molecule of any

one of claims 7 to 34 in a sample comprising utilizing direct agglutination methods, indirect antiglobulin tests, monoclonal anti-D antibodies and/or adsorbtion/elution techniques.

67. The method of any one of claims 35 to 66 wherein said sample is blood, serum, plasma, fetal tissue, saliva, urine, mucosal tissue, mucus, vaginal tissue, fetal tissue obtained from the vagina, skin, hair, hair follicle or another human tissue.

68. The method of claim 67 comprising enrichment of fetal cells or extraction of fetal DNA or mRNA from maternal tissue, like peripheral blood, serum or plasma.

69. The method of any one of claims 35 to 68 wherein said nucleic acid molecule or proteinaceous material from said sample is fixed to a solid support.

70. The method of claim 69 wherein said solid support is a chip.

71. Use of the nucleic acid molecular structure or the nucleic acid molecule of any one of claims 1 to 34 for the analysis of a negative or a positive Rhesus D phenotype.

72. Use of the nucleic acid molecular structure or the nucleic acid molecule of any one of claims 1 to 34, the vector of claim 44 or the protein product of the *RHD* gene of claim 47 for the assessment of the affinity, avidity and/or reactivity of monoclonal antibodies or of polyclonal antisera preferably anti-D antisera, anti-C antisera, anti-globulin or anti-human-globulin antisera.

73. Use of cells, preferably red blood cells from probands carrying the nucleic acid molecular structure or the nucleic acid molecule of any one of claims 1 to 34 for the assessment of the affinity, avidity and/or reactivity of monoclonal anti-D

or anti-C antibodies or of polyclonal anti-D or anti-C sera or of anti-globulin or of anti human globulin antisera or of preparations thereof.

74. A method for the characterization of the monoclonal antibodies or polyclonal antisera or of a preparation thereof said method comprising

(a) testing the nucleic acid molecular structure of a sample of a proband for the presence of a breakpoint or mutation as defined in any one of claims 1 to 34;

(b) correlating, on the basis of the mutation or deletion status and the allelic status of the *RHD* gene, the nucleic acid with the density of the protein product of the *RHD* gene on the surface of red blood cells of said proband;

(c) reacting said monoclonal antibodies or polyclonal antisera or said preparation thereof with a cell carrying the protein product of the *RHD* gene on its surface;

(d) characterizing said monoclonal antibodies or polyclonal antisera or said preparation thereof on the basis of the results obtained in step (c).

75. The method of claim 74 wherein said characterization comprises the determination of reactivity, sensitivity, avidity, affinity, specificity and/or other characteristics of antibodies and antisera.

76. The method of claim 74 or 75 wherein said cell carrying the protein product of the *RHD* gene on its surface is a red blood cell.

77. A method for determining whether a patient in need of a blood transfusion is to be transfused with RhD negative blood from a donor comprising the step of testing a sample from said patient for the presence of one or more nucleic acid molecular structures or nucleic acid molecules of any one of claims 2 to 5 and 7 to 34, wherein a positive testing for two different of said nucleic acid molecular structures is indicative of the need for a transfusion with Rh negative blood.

78. A method for determining whether blood of a donor is suitable for transfusion to a patient in need thereof who should not be exposed to antigen C comprising the step of testing a sample from said donor for the presence of the nucleic acid molecular structure of claim 11 wherein a positive testing for the nucleic acid molecular structure of claim 11 precludes the transfusion of the donor's blood.

79. A method for determining whether blood of a donor may be used for transfusion to a patient in need thereof comprising the step of testing a sample from said donor for the presence of one or more nucleic acid molecular structures or nucleic acid molecules of any one of claims 1-34, wherein a negative testing for the nucleic acid molecular structure of claim 2-5 with or without a negative testing for one or more nucleic acid molecular structures or nucleic acid molecules of claim 7-34 excludes the transfusion of the donor's blood to a patient that is typed as RhD negative.

80. A method of assessing of the risk of a RhD negative mother of conceiving or carrying an RhD positive fetus or of the risk of a mother having an anti-D titer of conceiving or carrying a fetus at risk to develop hemolytic disease of the newborn comprising assessing a sample obtained from the father of the fetus for the presence of one or more nucleic acid molecular structures or nucleic acid molecules as defined in any one of claims 1 to 34.

81. The method of claim 80 said nucleic acid molecular structure carryies mutations or deletions.

82. A method for assessing the possibility or likelihood of a man being the father of a child by assaying a sample obtained from said man for the presence of one or more nucleic acid molecular structures or nucleic acid molecules of any one of claims 1-34, wherein the test results are used to determine the homozygosity for, the heterozygosity for or the absence of any nucleic acid

molecular structure or a nucleic molecule of claims 2-5 and 7-34 used to infer the possibility or likelihood of said man being the father of the child.

83. A method for treating a pregnant woman being Rhesus D negative, wherein the foetus does not carry two nucleic acid molecular structures or nucleic acid molecules of any one of claims 2 to 5 and 7 to 34 or is not homozygous for any nucleic acid molecular structure of any one of claims 2 to 5 and 7 to 34 comprising administering anti D to said woman.

84. Use of an aptamer, phage, monoclonal antibody or a polyclonal antiserum as characterized in claim 49 or a preparation thereof for determination of the protein product of the *RHD* gene.

85. Use of claim 84 wherein said determination of the protein product of the *RHD* gene is effected in connection with blood group typing.

86. A preparation comprising the antibody or aptamer or phage of claims 49.

87. A method of identifying an antibody V_H or V_L chain or a combination thereof or an aptamer specifically binding to a protein product of the *RHD* gene of claim 47 comprising

(a) contacting the protein product of the *RHD* gene of claim 47 with a phage library displaying V_H or V_L chains or combinations thereof on the surface of the phage or with aptamers;

(b) identifying phage or aptamers that bind to said protein product; and optionally

(c) repeating steps (a) and (b) one or more times.

88. A method of identifying a monoclonal antibody specifically binding to a protein product of the *RHD* gene of claim 47 comprising

(a) contacting the protein product of claim 47 with one or more monoclonal antibodies;

(b) identifying monoclonal antibodies that bind to said protein; and optionally

(c) repeating steps (a) and (b) one or more times.

89. A method of identifying an antibody V_H or V_L chain or a combination thereof or an aptamer specifically binding to a protein product of the *RHD* gene of claim 47 comprising

(aa) contacting said protein product; and

(ab) a normal D polypeptide

wherein the normal D polypeptide is present in a molar mass that is higher, equal or less than the protein product of the *RHD* gene of (aa) with a phage library displaying V_H or V_L chains or combinations thereof on the surface of the phage or with aptamers;

(b) identifying phage or aptamers that bind to said protein product of the *RHD* gene of (aa); and optionally

(c) repeating steps (a) and (b) one or more times.

90. A method of identifying a monoclonal antibody specifically binding to a protein product of the *RHD* gene of claim 47 comprising

(aa) contacting the protein product of the *RHD* gene; and

(ab) a normal D polypeptide

wherein the normal D polypeptide is present in a molar mass which is higher, equal or less than the protein product of the *RHD* gene of (a) with one or more monoclonal antibodies;

(b) identifying monoclonal antibodies that bind to said protein product of the *RHD* gene of (a); and optionally

(c) repeating steps (a) and (b) one or more times.

91. The method of any one of claims 87 to 90, wherein the protein product of the *RHD* gene is exposed on the surface of a cell.

92. The method of any one of claims 87 to 91, wherein the polypeptide or host cell is affixed to a solid support.

93. The method of any one of claims 87 to 92, wherein subsequent to step (b) or (c), the following step is carried out:

(d) identifying the amino acid sequence of the V_H or V_L chains and/or identifying the nucleic acid sequence encoding said amino acid sequence.

94. The method of any one of claims 87 to 90, wherein, in the case that only one round of selection is employed for the identification, the number of protein molecules of the *RHD* gene of (a) is in molar excess over the number of phage particles.

95. Use of cells, preferably red blood cells comprising the protein product of the *RHD* gene of claim 47 or produced by the method of claim 46, from probands for the assessment of the affinity, avidity and/or reactivity of monoclonal anti-D or anti-C antibodies of claim 49 or of polyclonal anti-D or anti-C antisera or of anti-globulin or of anti-human-globulin antisera or of preparations thereof.

96. Use of *SMP1*-polymorphisms to determine specific *RH (RHD-RHCE)*-haplotypes genetically linked to said polymorphisms.

97. A method for detection of specific *RH (RHD-RHCE)*-haplotypes comprising the determination of *SMP1*-polymorphisms within the *SPM1* gene.

98. Kit comprising

(a) the oligonucleotide of claim 48; and/or

(b) the antibody of claim 49; and/or

(d) the aptamer of claim 49; and/or

(e) the phage of claim 49; and/or

(e) a pair of primers useful for carrying out the amplification reaction of any one of claims 54 to 61.

99. A process to determine the presence of an antigen C encoded by a *RHD* gene comprising the step of detecting any of the breakpoint regions mentioned in claim 11.

100. A process to determine the presence of an antigen C comprising the steps of the process of claim 39.

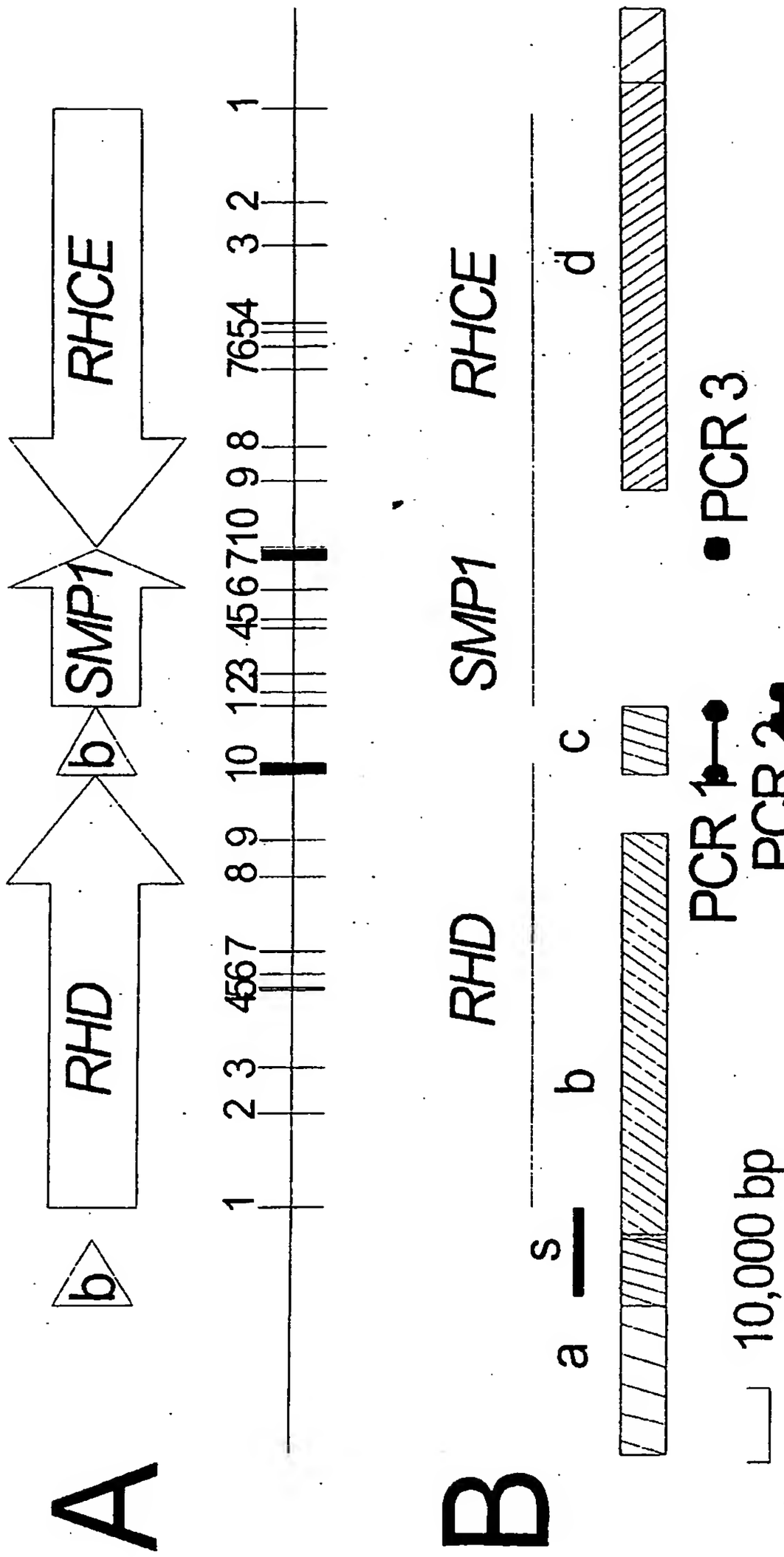
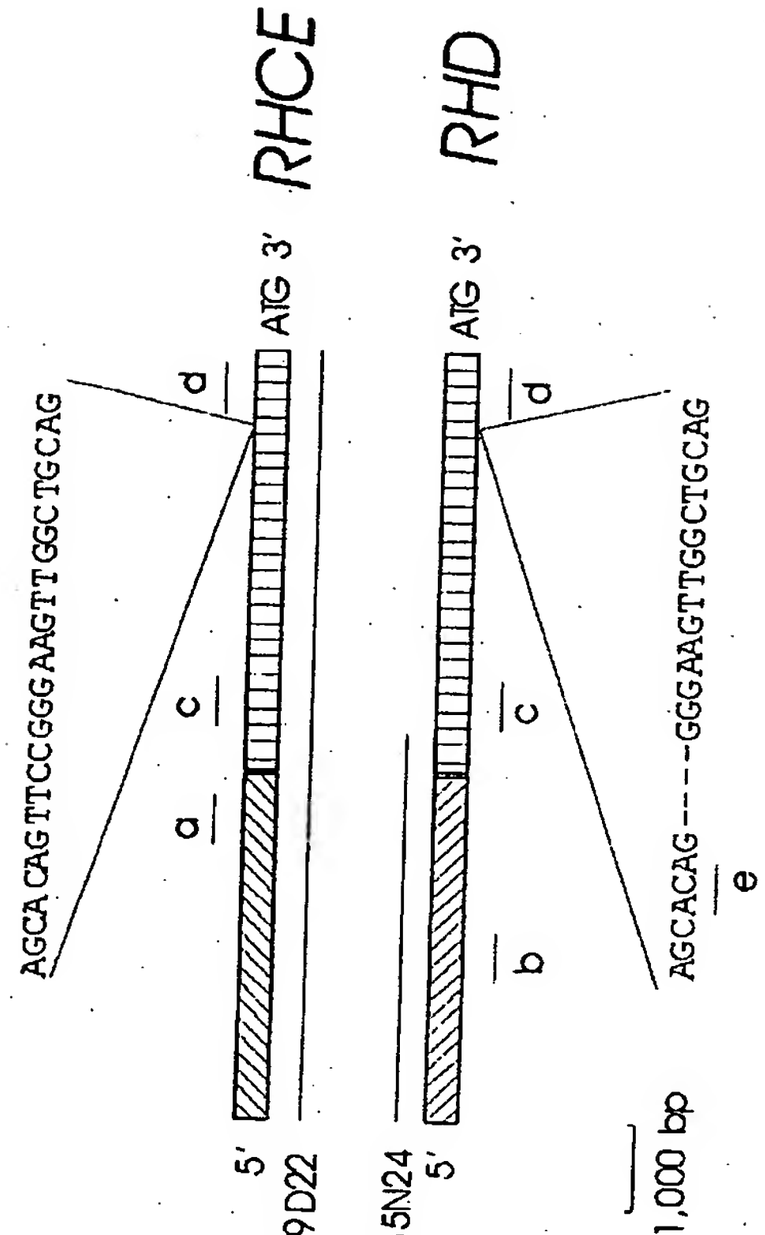

A
B
RHCE
SMP1
RHD
b
1 2 3 4 5 6 7 8 9 10
RHCE
SMP1
RHD
d
c
b
a
s
PCR 1
PCR 2
PCR 3
10,000 bp

Fig. 1

A


RHCE
RHD
AGCA CAG TTCCGGG AAGTTGGC TGCAG
a
c
d
ATG 3'
5'
dJ469D22
dJ465N24
b
AGCACAG----GGGAAGTTGGCTGCAG
e
1,000 bp

B

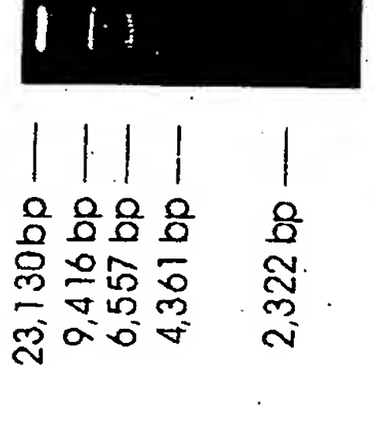

1 2 3 4 5 6 7 8 9
130 bp
416 bp
557 bp
361 bp
322 bp
Primers
RHD
23,
9,
6,
4,
2,

Fig. 2

Fig. 3

```
-- Rhesus box ------------------------------------>
gcatgcgcgactgagccgggtggatggtactgctgcatccgggtgtctg

                  <- SMP1 Exon 1 (91 bp, 5'UTR)-------
gaggctgtggccgttttgttttcttggctaaaatcgggggagtgaggcggg

-- SMP1 Exon 1 ----------><--- Intron ~ 2,000 bp
...actgcacgacggggctggactgacgt.....................

Intron --->&lt;---- SMP1 Exon 2 (106 bp, 93 translated)-----
.........agctgaaaaaaATGTCTGGATTTCTAGAGGGCTTGAGATGCTCAG...

-- SMP1 Exon 2 ----------><--- Intron ~ 2,500 bp
...GCTTCCATTGCTGCTGGTGTACTAgt...

Intron ---->&lt;--- SMP1 Exon 3 (113 bp, all translated)----
..........agTTTTTTACAGGCTGGTGGATTATCATAGATGCAGCTGTTATT...

-- SMP1 Exon 3 ----------><--- Intron ~ 8,000 bp
...TATAGCAACCATAGCCTTCCTAATgt.....................

Intron ---->&lt;--- SMP1 Exon 4 (68 bp, all translated)-----
..........agGATTAATGCAGTATCGAATGGACAAGTCCGAGGTGATAGTTA...

-- SMP1 Exon 4 ----------><--- Intron ~ 1,400 bp
...TGAAGGTTGTCTGGGTCAAAACAGgt.....................
```

Fig. 3 cont.

```
Intron ---->&lt;--- SMP1 Exon 5 (93 bp, all translated)-----
.........agGTGCTCGCATTTGGCTTTTCGTTGGTTTCATGTTGGCCTTTG...

-- SMP1 Exon 5 ----------><--- Intron ~ 4,000 bp
...TTTTTGGAGGTTATGTTGCTAAAGgt.....................

Intron ---->&lt;--- SMP1 Exon 6 (61 bp, all translated)-----
.........agAAAAAGACATAGTATACCCTGGAATTGCTGTATTTT.........

-- SMP1 Exon 6 ----------><--- Intron ~ 4,000 bp
...TCCAGAATGCCTTCATCTTTTTTGgt

Intron ---->&lt;--- SMP1 Exon 7 (1,703 bp, 47 translated)---
.........agGAGGGCTGGTTTTTAAGTTTGGCCGCACTGAAGACTTATGGCAGT

                    <---------- RHCE Exon 10 -----
-- SMP1 Exon 7 ------------------------------
GAacac....agcatcatcctaatgaaactaaacatttattttaaac

---------- RHCE Exon 10 ----------------------------
------- SMP1 Exon 7 --->
ttattaaattgactcttaaactaagttttagtctttaatttttaatatcaa

                                  >---- Homology with RHD -
-- RHCE Exon 10 ----------------------------------------
atctgtctctgaccttgtttcattatacataaggagctttgctgtcatgagcgtttc
```

Fig. 3 cont.

---------- Homology with *RHD* ----------
---------- *RHCE* Exon 10 ----------
tcacgtacaaatgcaggcaacagtgagaggaagttgtcttgtt

-- Homology with *RHD* ----------
-- *RHCE* Exon 10 ----------
tttgaacaggccttgtttttcttggatgcttttgcTTAAAATCCAACAGCCAAATGAGG

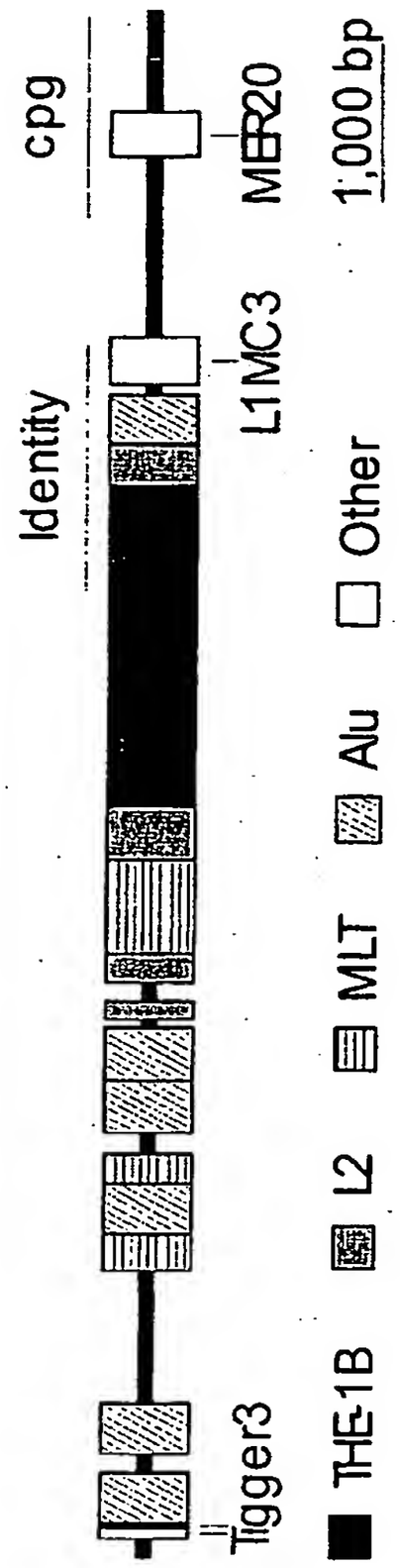

cpg
MER20
1,000 bp
Identity
L1MC3
Tigger3
THE1B
L2
MLT
Alu
Other

Fig. 4

Fig. 5

attcactatcacaagaacagcacgggtaa
****
-*******g**********

-------rez7---------->
gacctgtccccatgattcagttacct
****
****

cccactgggtccctcccacaacgcatgggaattcaggatgagattt
****
****

gggtggggacacaaccaaaccctatcattccacccatggcccctcccaaa
****
****

tttcatgtcctcacatttcaaaaccaatcacaccatcccaacagtccctc
****
****

aaagtcttaaatgatttcagcattaactcaaaagtccacagtctaatgtc
****
****

Fig. 5 cont.

```
tcatctgagacaaggcaagtcctttccatttatgagcctataaaatccaa
**************************************************
****************************c*********************

agcaagttagttacttcctagatacaatggggtacaggcattgggtaaa
**************************************************
*********a************************************

tacagccattccaaatgggataaattggtcaaaacaaagaggctacaggc
**************************************************
**************************************************

ccatgagagtccaaaatccagtggggcagtcaaatcttaaagctccaaaat
**************************************************
**************************************************

gatctcct-ttgactccacatctcacatccaggtcacgcagatggaagg
********_*****************************************
********c*****************************************

ggtgggttcccatggtcttgggcagctctgcccctgtacctttgcagggt
**************************************************
**************************************************
```

Fig. 5 cont.

```
acagcctccctctcagctgctttcatgggctggcattgagtgtctgcagc
**************************************************
**************************************************a*
                                                 ^^^^^^

ttttccaggtacacggtgcaagctgtcggtggatctaccattctggggtc
**************************************************
**************************************************

tggaggacctcttctcacagctccactaggtggtgccccagtagggactg
**************************************************
**************************************************

tgtgtggggtctctgaccccacatttcccttctgcactgccctggcagag
**************************************************
**************************************************

gatctccatgagggccctgctcctgcagcaaacttctgactgggcatcca
**************************************************
**********************c*****************c**********
                      ^^^^^^

ggcatttccgcacatcctctttaatctaggcgaaggtttccaaaccccaa
**************************************************
**************************************************g
```

Fig. 5 cont.

```
ttcttgacttctgtgcactcgcagtctcaacaccacatggaagctgtcaa
**************************************************
**************************************************

ggcttggggcttgcactccccgaagctacagcccaagctctaccttgcct
**************************************************
**************************************************

cccgtcagtcatggttgggagtggctgggatgcagggcaccaagtcccta
**************************************************
**t***********************************************

ggctgcacacagcatgaggaccccgggcctggccaacaaaaccatttttt
**************************************************
**************************************************

        +-- breakpoint region ->
        +-- identity region ->
cctgatacctctggacctgtgatgggaggggttgccataaagacctctga
**************************************************
*******t******************************************

catgccctggagacattttccccattgtcttgggaattagcatttggctc
**************************************************
**************************************************
```

Fig. 5 cont.

```
ctgttactcatgcaaatttctgcagccagcttgaatttctcctcagaaaa
**************************************************
**************************************************
                      ^^^^^^

tgggaatttttctttttctatcacattgtcaggctgcaaattttccgaactt
**************************************************
**************************************************

ttatgctctgcttcccttataaaactgaatgtctttaacagcacccaag
**************************************************
**************************************************

tcacctcttgaatgctttgctgcttagaaatttctcctgccagatactct
**************************************************
**************************************************

aaatcatctctctgaagttcaaagttctacaaatatctcgtgcaggggca
**************************************************
**************************************************

aaatgccgccagtatctttgctaaaacataacaagagtccccttgctcc
**************************************************
**************************************************
```

Fig. 5 cont.

agttcccaacaagttcctcatttccgtctgagaccacctcagcctatgga
****
****

ctttattgtccacagtgctatcagcattttgggcaaagccattcaacaag
****
****
tctctaggaagttccaaactttcccacatttgcctgtcttcttctgagcc
****
****

ctccaaactgttccaaaccctgcctgttacccagttccaaagtcacatac
****
****

ccatttttgagtatctacggcagcaccccactctactggtaccaatttag
****
****

ccactgaagtagttggagaacagaagtaatagactctggtttacattgta
****
****

aaagcttctctgtggctgctgtgtgaagaaaatatatgagaatgaagccc
****
****

Fig. 5 cont.

caagatgaagcagggacacagttgcagtggttagagtaagaaatgctgct
****
****

ggctggcactgaagtgatagcctggaggtttgtgtgtgcacatgcatgtg
****
****

tatgtgttttacgatagtaggcccaacagat
****
****

<-- breakpoint region ----+
actgtaatccacacttgttttttttttt----ga
**********tttt**
**********tttt**

gacagagtctcacctgttgcctagactagaatgc
****
****

agtggcacaatcttggctcactacaacctccacctcccaggttcaaacaatc
****
****

Fig. 5 cont.

cttgtgcttcagcctcccgagtagttgggattacaggtgtgtgccacc
****
****

gtgcccagctatatttttgtattttagcagagatgggattttgccacat
****
****

tggccaggctggtcttgaactcctggcctcaagcaatcctcccacctta
****
****

gcctcccaaagtgctgagccaccacacctggccgcaactgattttaatc
****
****

atgaaatgacacatacatttaaaaaacccaatacctataatattcctggc
****
****

tagtactcttcacatctatatcatcaaaaacaaagaaagtatgtgaaact
****
****

Fig. 5 cont.

```
gacacagccaaggggagactaaggagacataacaattaactgtaatgtgg
**************************************************
**************************************************

tattctggaggggatcctggaacagaaaaagacattaggcaaaaaactaa
**************************************************
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Fig. 5 cont.

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*******a**********g**********g*******

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****
****

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****

Fig. 5 cont.

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*****--*****************************

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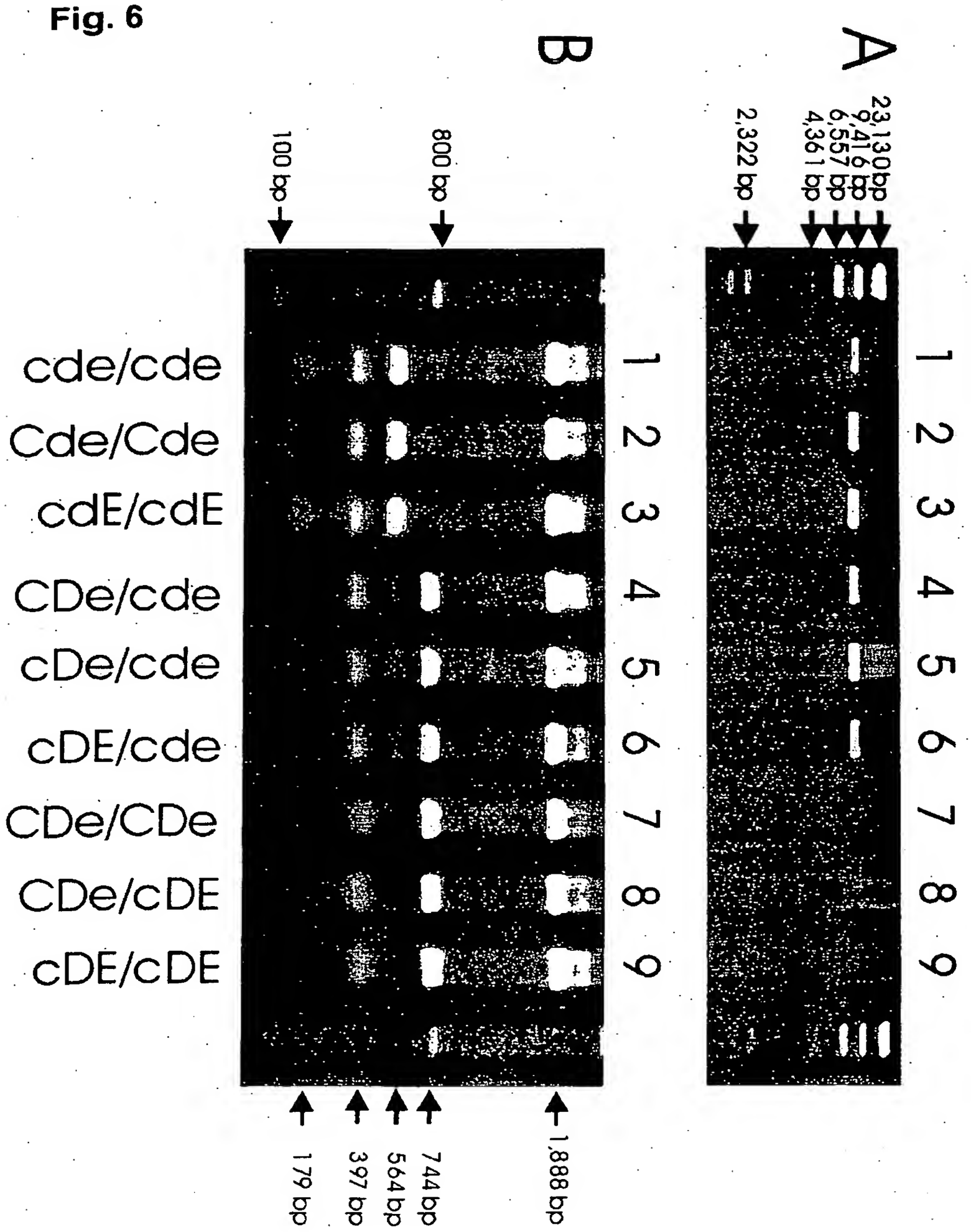
Fig. 6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23,130bp
1 2 3 4 5 6 7 8 9
1,888 bp

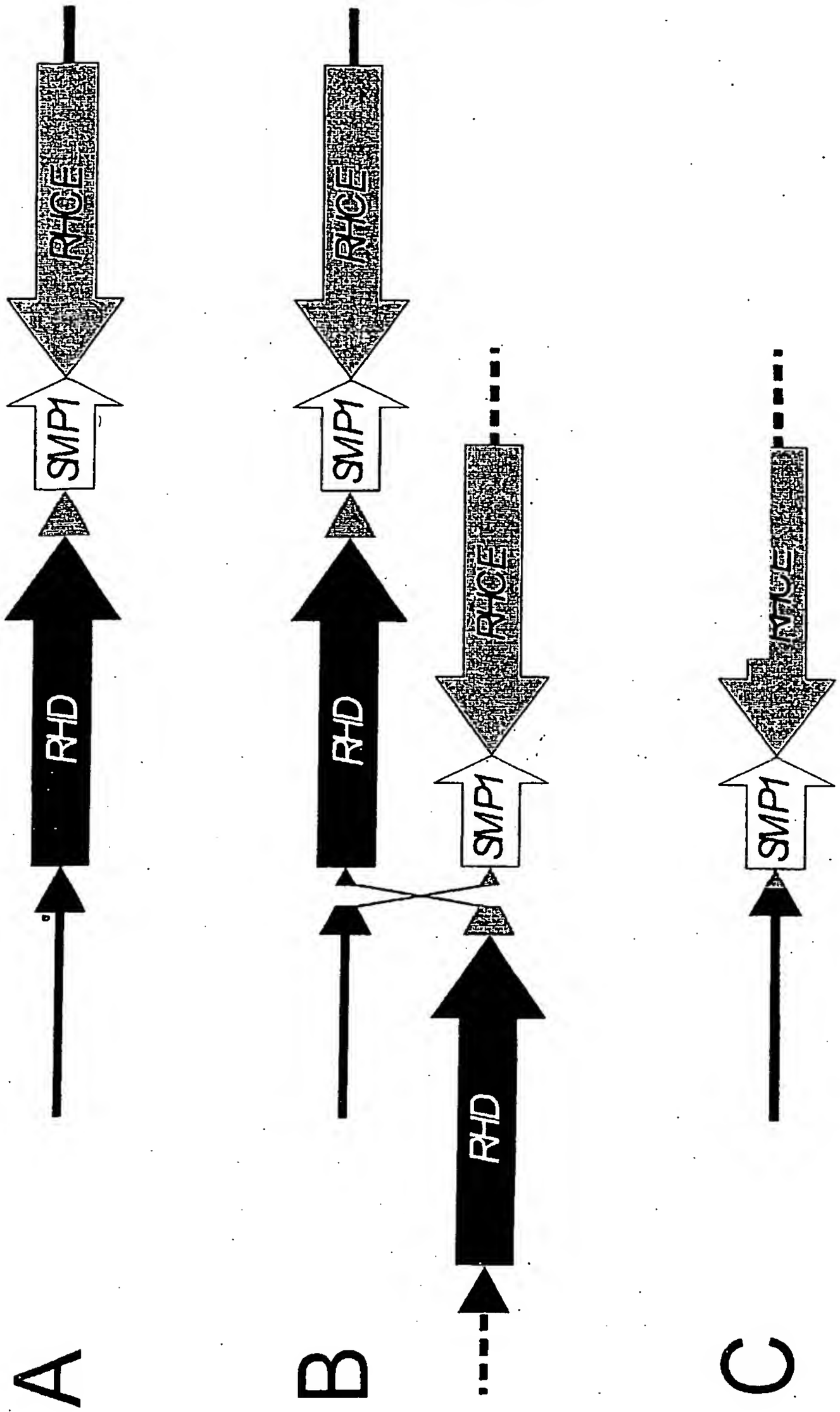

Fig. 7

Fig. 8

Hybrid Rhesus box of RHD negatives

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(start of Rhesus box)
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Fig. 8 cont.

Hybrid Rhesus box of RHD negatives

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Fig. 8 cont.

Hybrid Rhesus box of RHD negatives

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Fig. 8 cont.

Hybrid Rhesus box of RHD negatives

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(end of Rhesus box)
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3'

Fig. 9

Upstream Rhesus box of D-positives

5' ctagaaaacactttgtcattttagaggtgtta
(start of Rhesus box)
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Fig. 9 cont.

Upstream Rhesus box of D-positives

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Fig. 9 cont.

Upstream Rhesus box of D-positives

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cgagacccaaactgacggcttctagaagaggggcgagcccggccgcaagtctttcacgtagc

Fig. 9 cont.

Upstream Rhesus box of D-positives

taagtcatcgttgcttccggcttcttaccgttctccccctttgtaaacggttacctcccgaaa
acccaggctctcctccaacagtggttctcaagcgaggcgatcttccccgggaggggatattt
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gggggatgcagctacacaacctgcgaagcacgggacagcaccctccccaacccagacagaat
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gacgggtacgaaggccaacggacgccttcctttagaacgctcagcacacagagcaacttctc
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gcggcggaaagcccctgaacccagcgcccgggcatgcgcagacgcgttgttgtggtgggcgt
ggctccctccggacccggcgccccgccctccgccccgtgtccgcatgcgcgactgagccgcg
ggggtggtactgctgcatccgggtgtctg
(end of Rhesus box)
aagatccgatgaaataacatatgcaaaatgattgggtccgtgattggcattccagaaatgg
3'

Fig. 10

Upstream Rhesus box of D-positives

5' ctagaaaacactttgtcattttagaggtgtta
(start of Rhesus box)
tccaatgttcgcgcaggcactggagtcagagaaaatggagttgaatcctttctctgccactc
tttgaggagaatctcaccatttattatgcactgtagaatacaacaataaaatacagccatgt
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gacagagtctcactctgtcacccaggctggagtgcagtggtgcaatctcggctcactgcaat
ctccgcctcccaggttcacgccattctcttgcctcagtctcccgagtagctgggcctacagg

Fig. 10 cont.

Upstream Rhesus box of D-positives

cgcccgccaccctgcccagctaatttttgtatttttagtagagacggggtttcaccatgtt
agccaggatggtctcaatctcctgacctcgtgatccacctgcctcagcctcccaaagtgctg
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ggagtcttgctgtgttgcccagcctggagtgcagtgttatgattttggctcactgcaacctc
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Fig. 10 cont.

Upstream Rhesus box of D-positives

gatctccatgagggccctgctcctgcagcaaacttctgactgggcatccaggcatttccgca
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gcgcctccgctagggagactgcacgttgcgcctgtgcttcctgcggtggcgccttctgcaag

Fig. 10 cont.

Upstream Rhesus box of D-positives

gagacctcgaccctgctccctctccggggctggatctgactccttgacggtgattccagacg
cgagacccaaactgacggcttctagaagaggggcgagcccggccgcaagtctttcacgtagc
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acccaggctctcctccaacagtggttctcaagcgaggcgatcttccccgggaggggatattt
ggcaaagtctgggggcatttttggttcactggggctgctacttgcatccactgggtagaggc
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ggctccctccggacccggcgcccccgccctccgccccgtgtccgcatgcgcgactgagccgcg
ggggtggtactgctgcatccgggtgtctg
(end of Rhesus box)
aagatccgatgaaataacatatgcaaaatgattgggtccgtgattggcattccagaaatgg
3'

Fig. 11

```
ctgatctaca taggaattgt tttcaagaca tttctgcatt cctctagtga cagggtgctc      60
actacctcat gagtatttca gtggacaact gtaatggtca ataaagtatc cactttccac     120
ctccctgcag ctcctggccc tggctttatt ctctggggct ccacacattc agtttacact     180
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ctgcagggtt agaactaagt cccaagcccc ctaaagctca tgccagggga ctggactgtc     420
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caggcagagt cctgggctgc cctgtgagga ggtgaccaga ggtagagcaa cttcacccta     540
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agtaatttca actgtgtaac tatgaggagt cagttctacg tgggtcctat ctgtatcctc     720
cccagggctc agctccattc tttgctttca ttcattctca ttcaatacat tgttgttaag     780
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gcttccgtgt taactccata gagaggccag cacaaccagc cttgcagcct gagataaggc    1140
ctttggcggg tgtctccccc atcgctccct caagccctca agtaggtgtt ggagagaggg    1200
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```

Fig. 12

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2938 CTAGAGAGGGAAGTTTTTGAAAATTAAACACTGTCTAATTTTCTGCAAAGTTTTTATTCATGAATTAAGAGTATTTCCCTTAGTCCATTATTCCCAAGGC RHCE
     CTAGAGAGGGAAGTTTTTGAAAATTAAACACTGTCTAATTTTCTGCAAAGTTTTTATTCATGAATTAAGAGTATTTCCCTTTGTCCATTATTCCCAAGGC Cde^s
     CTAGAGAGGGAAGTTTTTGAAAATTAAACACTGTCTAATTTTCTGCAAAGTTTTTATTCATGAATTAAGAGTATTTCCCTTTGTCCATTATTCCCAAGGC RHD

                          |********************** breakpoint region ************************************
3038 AAATATGGAAGTTTGATCATATGCTAATCATACTAAAGCTGGATTCTCTTTAAGAGATTGAGAAATTAAAAGGCAAAAGCTGATATATCATGTTTAGTTA RHCE
     AAATATGGAAATTTGATCATGTACTAATCATACTAAAGCTGGATTCTCTTTAAGAGATTGAGAAATTAAAAGGCAAAAGCTGATATGTCATGTTTAGTTA Cde^s
     AAATATGGAAATTTGATCATGTACTAATCATAATAAAGCTGGATTCTCTTTAAGAGATTGAGAAATTAAAAGGCAAAAGCTGATATATCATGTTTAGTTA RHD

     ********************************** breakpoint region **********************|
3138 TACTGTGAGTCTTATAAGAAGCTGGGAGGCAACCCCATTAACTCACCAGAATACAGAACTCAGTCTCACAACTTAAATATAATTCCTCTCAAACCTTTTC RHCE
     TATTGTGAGTCTTATAAGAAGCTGGGAGGCAACCCCATTAACTCACCAGAATACAGAACTCAGTCTCACAACTTAAATATAATTCCTCTCAAACCTTTTC Cde^s
     TATTGTGAGTCTTATAAGAAGCTGGGAGGCAACCCCATTAACTCACCAGAATACAGAACTCAGTCTCACAACTTAGATATAATTCCTCTCAAACCTTTTC RHD

3238 CTCAAAG-TTAAATTCTGAAAATAATCTTGTGATTAAGAGAAGAAGGCTGTCCACCAATGGACTTATCTGTTATTTCTTCCTTATTGTGAGCTTAATGGC RHCE
     CTCAAAG-TTAAATTCTGAAAATAATCTTGTGATTAAGAGAAGAAGGCTGTCCACCAATGGACTTATCTGTTATTTCTTCCTTATTGTGAGCTTAATGGC Cde^s
     CTCAAAGATTAAATTCTGAAAATAATCTTGTGATTAAGAGAAGAAGGCTGTCCACCAATGGGCTTATCTGTTATTTCTTCCTTATTGTGAGCTTAATGGC RHD

3337 ATGACAAAGCAGAGGCAAAGAGGCATACATCAATTCTTCAAAGTAGGAAGTCAAAAAGGTCAGAGCTTCCACAGCATGGCAACAGCTTTGCAGATGCCCA RHCE
     ATGACAAAGCAGAGGCAAAGAGGCATACATCAATTCTTCAAAGTAGGAAGTCAAAAAGGTCAGAGCTTCCACAGCATGGCAACAGCTTTGCAGATGCCCA Cde^s
     ATGACAAAGCAGAGGCAAAGAGGCATACATCAATTCTTCAAAGTAGGAAGTCAAAAAGGTCAGAGCTTCCACAGCATGGCAACAGCTTTGCAGATGCCCA RHD

3437 CATCGTGATAGTTGAAATAGCAAAGCCCAGCAAAGGTTAAAGCTGAAAATGCCAAAAGCCCTGCCTTGGCAGCTTTCTGCGAGGCATCCCCATGAACATA RHCE
     CATCGTGATAGTTGAAATAGCAAAGCCCAGCAAAGGTTAAAGCTGAAAATGCCAAAAGCCCTGCCTTGGCAGCTTTCTGCGAGGCATCCCCATGAACATA Cde^s
     CATCGTGATAGTTGAAATAGCAAAGCCCAGCAAAGGTTAAAGCTGAAAATGCCAAAAGCCCTGCCTTGGCAGCTTTCTGCGAGGCATCCCCATGAACATA RHD

3537 GTCAGTAACAACTTGTCCAAGGCCCCAGTGACCATGAAGAGTGAGGGCTGCAGCCAGGGAATAGTCCGTCGCAGAGCAAGGATTCAAATAAGCAGCCGGA RHCE
     GTCAGTAACAACTTGTCCAAGGCCCCAGTGACCATGAAGAGTGAGGGCTGCAGCCAGGGAATAGTCCGTCGCAGAGCAAGGATTCAAATAAGCAGCCGGA Cde^s
     ATCAGTAACAACTTGTTCAAGGCCCCAGTGACCATGAAGAGTGAGGGCTGCAGCC-GGGAATAGTCCGTCGCAGAGCAAGGATTCAAATAAGCAGCCGGA RHD
```

Fig. 13

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2726 TTGTATCTCTTTTTACAGCTACCTCCCATTTCCCTTCTATTTCAAGCTAGTAACACAGTTTTCTTTTAAATTCATTTATTTAAATGTAAAAATAAGTCTA RHCE
     TTGTATCTCTTTTTACAGCTACCTCCCATTTCCCTTCTATTTCAAGCTAGTAACACAGTTTTCTTTTAAATTCATTTATTTAAATGTAAAAATAAGTCTA Cde^s
     TTGTATCTCTTTTTACAGCTACCTCCCATTTCCCTTCTATTTCAAGCTAGTAACTCAGTTTTCTTTTAAATTCAATTATTTAAATGTAAAAATAAGTCTA RHD

2826 TTTGGAGAAAAAAAATTTTTAATAGCATCTCTGGAATGCCAGTATGGCTAAATTCATGAATGTTGTCCTCAAATGCTGAAATCTGGGAAGCATCTGGCCA RHCE
     TTTGGAGAAAAAAAATTTTTAATAGCATCTCTGGAAGGCCAGTATGGCTAAATTCATGAATGTTGTCCTCAAATGCTGAAATCTGGGAAGCATCTGGCCA Cde^s
     TTTGGAGAAAAAAAATTTT-AATAGCATCTCTGGAATGCCAGTATGGCTAAATTCATGAATGTTGTCCTCAAATGCTGAAATCTGGGAAGCATCTGGCCA RHD

                                                          |************** breakpoint region ***************
2926 AGCTTTGTGGACAGGCCTGCCTAGTTTGAATCCCAAGAGCCACTCATTCCGAGCCACAAAACATTGGAATTCTTGGTTCACTTCCCTAACCTGAACTTGT RHCE
     AGCTTTGTGGACAGGCCTGCCTAGTTTGAATCCCAAGAGCCACTCATTCCGAGCCACAAAACATTGGAATTCTTGGTTCACTTCCCTAACCTGAACTTGC Cde^s
     AGCTTTGTGGACAGGCCTGCCTAGTTTGAATCCCAAGAGCCACCCAGTCCAAGCCACAAAACATTGGAATTCTTGGTTCACTTCCCTAACCTGAACTTGC RHD

     ********************************** breakpoint region ************************************************
3026 CCTCTGTGAAATAGGGACATTAATAGCTCACTCACAGGGCTGCTGTGAGGACATGTGTTGAGCTGAGGGTCTCGCCAGGGGAGACCCTGTGCAGGGAGAC RHCE
     CCTCTGTGAAATAGGGACATTAATAGCTCACTCACAGGGCTGCTGTGAGGACATGTGTTGAGCTGAGGGTCTGGTCAGGGGAGACCCTGTGCAGGGAGAC Cde^s
     CCTCTGTGAAATAGGGACACTAATAGCTCACTCACAGGGCTGCTGTGAGGACATGTGTTGAGCTGAGGGTCTCGCCAGGGGAGACCCTGTGCAGGGAGAC RHD

     ********************************** breakpoint region ************************************************
3126 TGTTATCATGGTGATGGATTTCTGCTTCATTCATTTCTTTTTCCAGACAGCATCATATAGAATGAGTTGTGGGGTGGCAGTCAGCAGGTTTGGGTTTATC RHCE
     TGTTATCATGGTGATGGATTTCTGCTTCATTCATTTCTTTTTCCAGACAGCATCATATAGAATGAGTTGTGGGGTGGCAGTCAGCAGGTTTGGGTTTATC Cde^s
     TGTTATCATGGTGATGGATTTCTGCTTCATTCATTTCTTTTTCCAGACAGCATCATATAGAATGAGTTGTGGGGTGGCAGTCAGCAGGTTTGGGTTTATC RHD

     ********************************** breakpoint region ************************************************
3226 CTCTATTCTGCCACTTATTACTTAAAAAAA------AAAACCCAACTTATATAGTATAAGCTATATCCAGAAAAGTGCAAATATCATACAAGTACCATTT RHCE
     CTCTATTCTGCCACTTATTACTTAAAAAAACCCCAAAAAAACCCAACTTATATAGTATAAGCTATATCCAGAAAAGTGCAAATATCATACAAGTACCATTT Cde^s
     CTCTATTCTGCCACTTATTACTTAAAAAAACCCCAAAAAACCCAACTTATATAGTATAAGCTATATCCAGAAAAGTGCAAATATCATACAAGTACCATTT RHD
```

Fig. 13 cont.

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     ********************************** breakpoint region ***********************************************
3320 GATGAATCTTCTGATATCCCCACATAACCAACACCCAGAACCTCTTCTTGTCTCATTCCAGGATAACCACTAACCTGACTTCTAACAGCATCAGTCAGTT RHCE
     GATGAATCTTCTGATATCCCCACATAACCAACACCCAGAACCTCTTCTTGTCTCATTCCAGGATAACCACTAACCTGACTTCTAACAGCATCAGTCAGTT Cde^s
     GATGAATCTTCTGATATCCCCACATAACCAACACCCAGAACCTCTTCTTGTCTCATTCCAGGATAACCACTAACCTGACTTCTAACAGCATCAGTCAGTT RHD

     ********************************** breakpoint region ***********************************************
3420 TTGTCTGTTTTTGTACATTATATATGTGATGGTTTGAATGTGTCCCCCAAATTTCATGTGCTAGAAACTTAATCCTTCAATTCATATGTTGATGCTATTT RHCE
     TTGTCTGTTTTTGTACATTATATATGTGATGGTTTGAATGTGTCCCCCAAATTTCATGTGCTGGAAACTTAATCCTTCAATTCATATGTTGATGCTATTT Cde^s
     TTGTCTGTTTTTGTACATTATATATGTGATGGTTTGAATGTGTCCCCCAAATTTCATGTGCTGGAAACTTAATCCTTCAATTCATATGTTGATGGTTTTT RHD

     ********************************** breakpoint region ***********************************************
3520 GGAGGAAGGGCCTTTGGGAAGTAATTAGGATTAGATAAGGTCATGGGGTGAGGTATGATGGCACTGGTGACTTATAAGAAGAGAAAGAGAAATCTGAGCT RHCE
     GGAGGAAGGGCCTTTGGGAAGTAATTAGGATTAGATAAGGTCATGGGGTGAGGTATGATGGCACTGGTGACTTATAAGAAGAGAAAGAGAAATCTGAGCT Cde^s
     GGAGGAAGGGCCTTTGGGAAGTAATTAGGATTAGATAAGGTCATGGGGTGAGGTATGATGGCACTGGTGACTTATAAGAAGAGAAAGAGAAATCTGAGCT RHD

     * breakpoint region *|
3620 GGCATGCTCTTGCCCTCTCACCGTGTGATGACTTCTCCATGTCATGATGCAGCAAGAAGGCCCTCACCAGATGGTGGCACCATGCTTTTGGACTTCCCAG RHCE
     GGCATGCTCTTGCCCTCTCACTGTGTGATGACTTCTCCATGTCATGATGCAGCAAGAAGGCCCTCACCAGATGGTGGCACCATGCTTTTGGACTTCCCAG Cde^s
     GGCATGCTCTTGCCCTCTCACTGTGTGATGACTTCTCCATGTCATGATGCAGCAAGAAGGCCCTCACCAGATGGTGGCACCATGCTTTTGGACTTCCCAG RHD
```
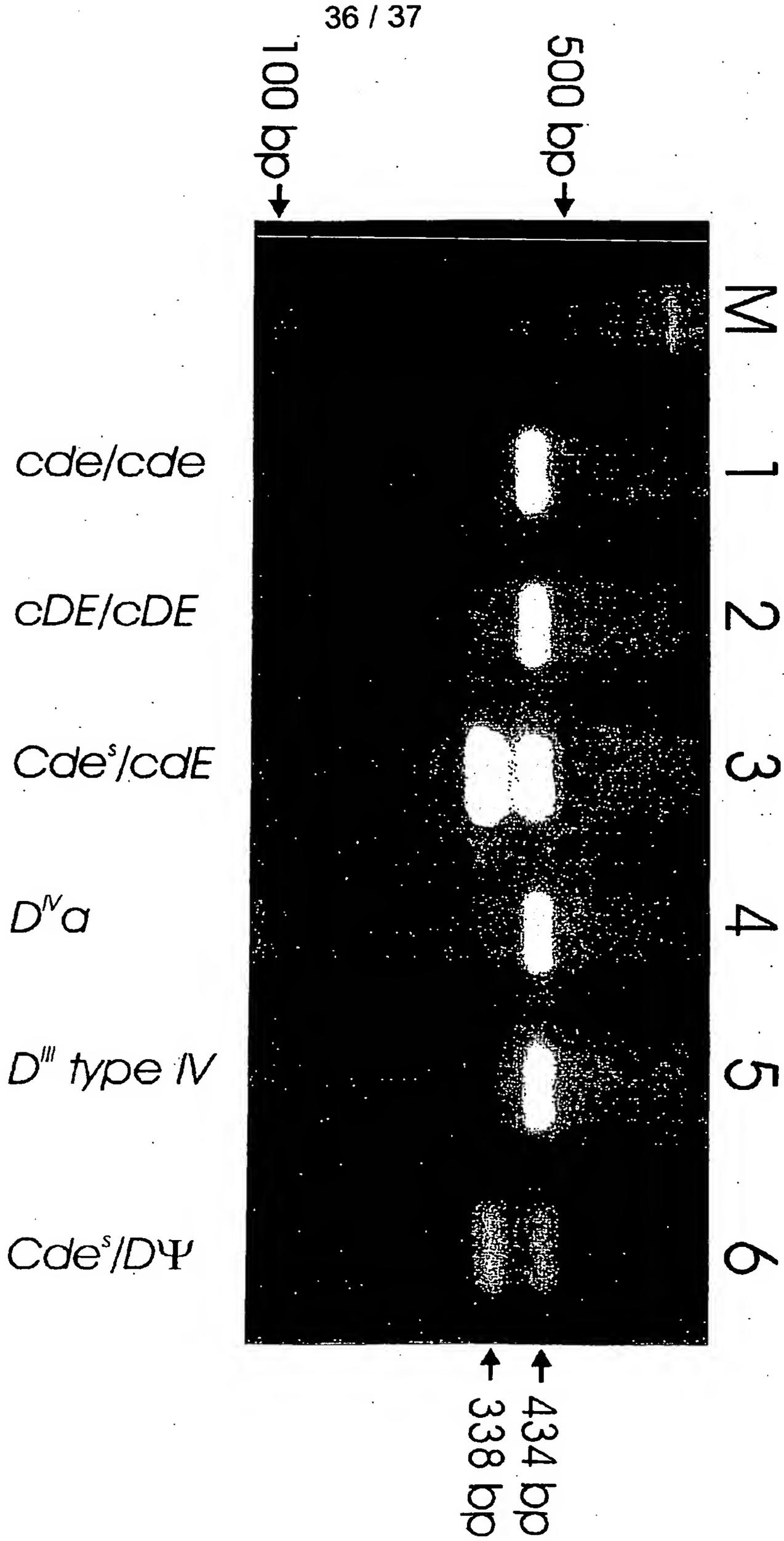

Fig. 14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2
3
4
5
6
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338 bp
434 bp

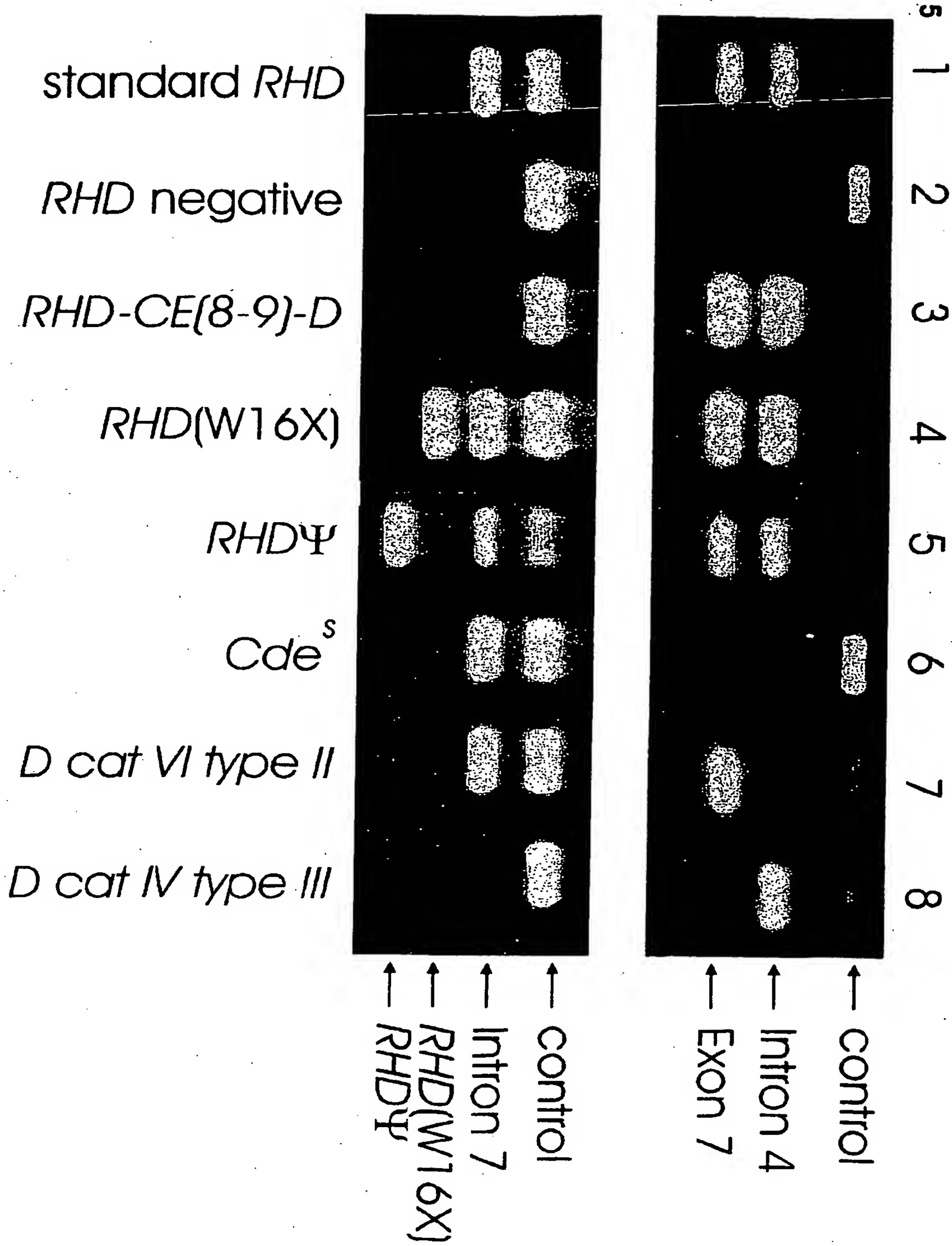

Fig. 15